Phytoremediative potential of *Brassica napus* L. - heavy metal tolerance and accumulation, phytochelatin system response and γ-glutamylcysteine synthetase genetic background

Przemysław Nuc, Konrad Wasinkiewicz, Katarzyna L. Janczur, Barbara Tomaszewska

Department of Biochemistry, Adam Mickiewicz University, Poznań, 61-614 Poland
Email: kjanczur@amu.edu.pl

Abstract

Worldwide heavy metal contamination causes severe hazard to ecological balance of ecosystems and to human health. Plants are more and more often used for decontamination of soils from heavy metals (HM) through phytoremediation processes what initiates a demand for heavy metals highly tolerant and hiperaccumulating plants. Those abilities are underlain by a variety of defense mechanisms activated by the plant in response to stress. Phytochelatin system's non-protein thiols (NPT): glutathione (GSH) and phytochelatins (PCs) participate in heavy metals ions chelation and sequestration. During phytochelatin system's response to HM stress PCs - short cysteine rich ions binding peptides - are nonribosomally synthesised from GSH with γ-glutamylcysteine synthetase (γ-ECS) acting as a rate limiting enzyme of the pathway. *Brassica napus* line DH O-120 phytoremediative potential has been studied through examination of metal accumulation and phytochelatin system performance in response to lead stress. Lead content of 44375.2 mg/kg d.w. in case of 1 mM and 48316.77 mg/kg d.w. in case of 3 mM $Pb(NO_3)_2$ applied, exceeding lethal values in experiments with other plants proved the high HM tolerance of *Brassica napus*. However, as 99.8% of metal was located in the roots, lead translocation to aboveground parts appeared to be low. The translocation barrier has been overcome by EDTA addition increasing lead content in the shoot up to 18% in case of 3 mM $Pb(NO_3)_2$. The dynamic PCs and GSH biosynthesis has been observed in *Brassica napus* seedlings and roots with a maximum NPT content in 24th hour of exposition propotional to lead concentration. RT-PCR and real time PCR examined γ-ECS gene (*GSH1*) transcription level increased in both roots and shoots and was well correlated with the enzyme activity increase. *Brassica napus* γ-ECS gene sequence has been determined. 15 exons locations within the gene have been predicted basing on *A. thaliana GSH1* structure and the sequence similar in 74% to *B. juncea* γ-ECS gene promoter has been determined as putative promoter region. Since *Brassicaceae* family is known for diversity within *GSH1* gene the first exon area has been RT-PCR studied and showed the variability of 9 nucleotide deletion presence indicating at least two distinct alleles of γ-ECS gene occurrence in *Brassica napus* genome. Relatively high biomass production, heavy metals tolerance and accumulation of oilseed rape as well as proven commercial phytoremediation performance of its relative species *B. juncea* makes the plant a good phytoremediation prospect. Close relationship to *A. thaliana* facilitating molecular analysis enable the transgenic approach in producing plants for phytoremediation. Optimal cultivars could be obtained through genetic modifications within phytochelatins biosynthetic pathway, e.g. γ-ECS overexpression.

Key words: *Brassica napus*, phytoremediation, lead, phytochelatin system, γ-glutamylcysteine synthetase

Introduction

The constant increase of worldwide occurring contamination with heavy metals may be well illustrated by lead content jump of Grenland's glacier from 0.05 μg/l to 0.5 μg/l within the past century. Non-essential heavy metals: cadmium and lead are the most toxic to all organisms. Since, besides the negative health effects typical for heavy metals, the prolonged exposition to lead causes mental retardation the metal is said to be a number one threat to children health. Heavy metals are extremly persistent in the environment and in living organisms (half life of lead introduced into the soil of modearate climat reaches up to 10 000 years), therefore their content biomagnificates within the trophic chain presenting a threat to human health through plant and animal food production around polluted areas. Decontamination is thus of crucial importance. In opposite to conventional methods, phytoremediation exploiting living plants for this purpose presents a group of cost-effective, environmentally friendly technologies. Among various phytoremediation processes used for pollutants removal phytoextraction, technique underlain by passive ions uptake with transpiration water flow and accumulation in plant tissues, is especially applicable in case of heavy metlas. The heavy metals hiperaccumulating and highly tolerant plants required might be obtained e.g. through genetic engineering. *Brassicaceae* is prooved to embrace the highest numebr of good heavy metals accumulators among all plant families. *Brassica juncea* has beed succesfully applied for phytoremediation of lead. Its closest relative - *Brassica napus* could possibly be similarly used, especially as the plant is known to be a good lead accumulator. Not only natural *Brassica napus* seems to be a good phytoremediation prospect but as genetically modified rapeseed's acrage comprises within five highest for world's transgenic crops it also presents a promising object for genetic engineering approach to obtain the plants with enhanced phytoremediative potential. Phytochelatin system - one of defense mechanisms activated by the plant under metal stress participatig in heavy metal ions chelation and sequestration serves as potential target for such genetic modifications. In response to heavy metal stress phytochelatin system's enzymes: γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) increase the rate of glutathione production. Then phytochelatines, short

cysteine rich peptides are non-ribosomally synthesised from glutathione precursor by phytochelatin synthase (PCS) to bind heavy metal ions. The process of phytochelatines synthesis is strongly limited by glutathione availability and γ-ECS catalizing glutathine precursor synthesis is therefore the rate limiting enzyme of the pathway and simoultanously the most interesting target for genetic modifications. As phytochelatin system's contribution to the overall plant response to heavy metal stress differs among plant species its performance in *Brassica napus* has been estimated by γ-ECS activity changes and non-protein thiols synthesis survey under lead stress. γ-ECS gene has been sequenced and structurally analysed and its two alleles have been detected to complete information about its possible gene level activation in response to heavy metal stress. Phytoremediative potential of natural *Brassica napus* has been estimated through lead tolerance, accumulation and accumulation enhanced by EDTA study.

Materials and methods

For lead accumulation, tolerance and γ-ECS mRNA accumulation assay *Brassica napus* plants were germinated and grown in perlit for 14 days using Hoagland medium. Lead was applied at two different concentrations of 1 mM $Pb(NO_3)_2$ and 3 mM $Pb(NO_3)_2$. The addition of 1 and 3 mM EDTA was used to study the influence of chelator on Pb transportation from roots to aboveground rapeseed organs. Lead content of *Brassica napus* leaf and root tissue was assayed with AAS technique described by Piechalak *et al*., 2002. Non-protein thiol compounds synthesis in seedlings grown on blotting paper saturated with different lead solutions was assayed by content analysis with HPLC method, combined with postcolumn reaction with Elmann reagent (Tomaszewska *et al.*, 1996). γ-ECS mRNA accumulation in response to lead stress was RT-PCR surveyed applying cyclophilin gene for γ-ECS cDNA amount standarizatrion and the results were confirmed using *real-time* PCR technique. Method by Orłowski M. and Meister A., 1971 was applied for lead stress triggered γ-ECS activity changes examination. *Brassica napus* genome fragment containing γ-ECS gene (*GSH1*) reading frame and promoter elements was obtained through John Innes Centre rapeseed BAC library screening with *A. thaliana GSH1* sequence based hybridization probe. *Brassica napus GSH1* full sequence was determined initially using primers pair complementary to sequence from *A. thaliana.* The structural analysis with Vector NTI software was also performed basing on *A. thaliana GSH1* sequence. *Brassica napus* γ-ECS gene alleles occurence was established with RT-PCR assey within the predicted first exon area on genomic DNA and cDNA obtained from total RNA derived from DH O-120 rapeseed plants.

Results and discussion

Lead tolerance, accumulation and EDTA-enhanced accumulation in Brassica napus *plants*

In lead stressed *Brassica napus* plants the heavy metal accumulation was proportional to the lead concentrationa applied - twice higher for 3 mM $Pb(NO_3)_2$ than for 1 mM $Pb(NO_3)_2$. The highest Pb content in the whole plant reached up to 44375 mg/kg DW for 1 mM $Pb(NO_3)_2$ stressed plants and 95374 mg/kg DW for 3 mM $Pb(NO_3)_2$ stressed plants. Lead content of plants treated with 1 mM increased during the whole experiment time while 3 mM $Pb(NO_3)_2$ stressed plants lead content decreased after reaching the top value after 48 hours. The drop was most probably caused by plant's tolerance threshold exceeding and the content leak from lead damaged cells and resulted in similar final lead content value in the seventh day of experiment for both 1 mM and 3 mM $Pb(NO_3)_2$ treated plants. Such accumulation values place *Brassica napus* in the group of good lead accumulators and remain in accordance with results presented by Wierzbicka, 1999 whose experiments proved rapeseed's highest lead accumulation among 22 plant species tested. 99.8% of lead was accumulated in *Brassica napus* roots for both stress factor concentrations applied. Lead ions absorbed from 1 mM $Pb(NO_3)_2$ solution appeared in the shoot on the fifth day of exposition and ions absorbed from 3 mM $Pb(NO_3)_2$ after 24 hours. Root is thus the main lead accumulation organ of rapeseed and the strong heavy metal ions translocation to aboveground parts barrier occurs what was previously confirmed by a number of authors (e.g. Kumar *et al*. 1995).

Tabela I Heavy metal content in rapeseed plants treated with lead up to 7 days.

days	lead content in rapeseed plants [mg/kg DW]			
	1 mM Pb		3 mM Pb	
	roots	shoots	roots	shoots
0	126	8,72	126	8,72
1	25032	6,59	47333	23,2
2	28320	6,36	95333	40,87
5	34090	54,91	75692	19
7	44320	55,2	48300	16,77

EDTA (ethylenediaminetetraacetic acid) is known to increase lead content in treated plants and to facilitate its transport to aboveground parts the most among all other synthetic chelators such as: CDTA, DTPA, EGTA etc. Pb-EDTA complex is immobilized by cell walls to much lesser extent than lead ions. As equimolar solutions of Pb and EDTA was previously proved by Piechalak *et al*., 2003. to most effectively perform the addition of 1 mM EDTA was used along with 1 mM $Pb(NO_3)_2$ and 3 mM EDTA along with 3 mM $Pb(NO_3)_2$. EDTA addition resulted in advantageous from phytoremediation viewpoint multi-fold increase of lead content in shoots from 0.2% of total lead accumulated by a plant without EDTA addition to 11 and 18% for 1mM EDTA-1 mM $Pb(NO_3)_2$ and 3 mM EDTA-3 mM $Pb(NO_3)_2$ stressed plants, respectively (Fig. 1).

However for all plants the unexpected total lead accumulation drop was observed - heavy metal content was 4 times lower in 1 mM $Pb(NO_3)_2$ and 1.6 times lower in 3 mM $Pb(NO_3)_2$ treated plants. EDTA usage for induced-phytoremediation is thus controversial especially that when applied to the soil it not only mobilizes metal ions for plants but also enables them to penetrate into deeper ground levels. The heavy metal ions phytoavalability enhancement could be possibly achieved by selected symbiotic rhizosphere microorganisms performance in stead of synthetic chelating chemicals usage.

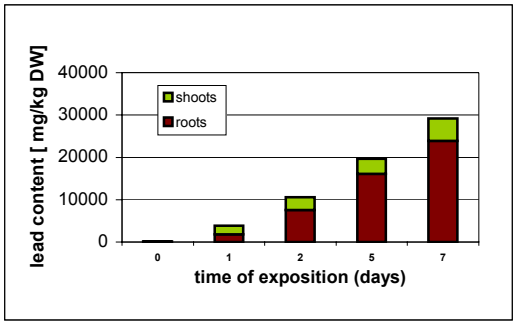


Fig. 1 Distribution of lead accumulated by 3 mM $Pb(NO_3)_2$-3 mM EDTA *B. napus* plants

The toxication symptoms [roots growth inhibition only in case of 1 mM $Pb(NO_3)_2$, leaves colour change, shoots growth inhibition, chlorosis and necrosis, roots turning brown, lateral roots growth inhibition in case of 3 mM $Pb(NO_3)_2$] were observed on plants treated with all stressing solutions. Especially intensive toxication symptoms appeared on Pb-EDTA stressed plants most probably as a reason of toxic influence of EDTA itself. Application of 1 mM $Pb(NO_3)_2$ solution guarantees lead ions influence on plants orders of magnitude higher than expected in the most contaminated soil solution in the environment. In case of this concentration the treated plants appearance still did not significantly differ from control plants. Regarding *Brassica napus* plants good shape assisted by accumulation of over 30 000 mg/ kg. DW exceeding lethal values in the experiments with other plants rapeseed lead tolerance might be characterized as relatively high.

Brassica napus phytochelatin system performance in response to lead stress

Glutathione (GSH) and phytochelatins (PCs), the phytochelatin system's non-protein thiols (NPT) are

known to participate in heavy metal detoxification by ions chelation. Non-protein thiol compounds synthesis in response to lead stress was observed in *Brassica napus* seedlings. Their content in plant tissue was proportional to stress factor intensity (Fig. 2). what confirms the phytochelatin system function in response to lead stress in rapeseed plants. In case of 14-day-old plants the NPT synthesis occurred in the roots only. NPT amount significantly increased after 6 hours of 3 mM $Pb(NO_3)_2$ and 18 hours later for 1 mM $Pb(NO_3)_2$. The NPT content decrease after second day of exposition suggests the temporary phytochlatin system's involvement in overall heavy metal detoxification. NPT synthesis observed in *Brassica napus* might serve as quick short term response to lead ions stress.

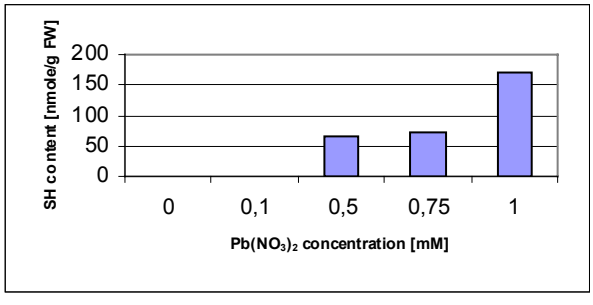


Fig. 2 Total non protein thiols in *B. napus* seedlings grown for 5 days with different lead concentrations

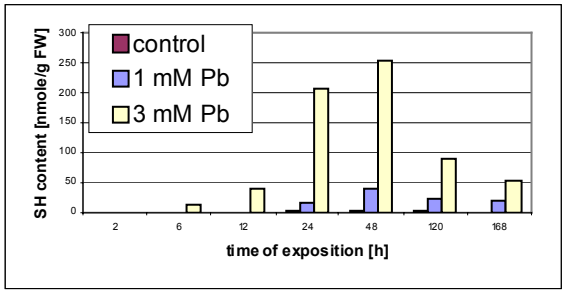


Fig. 3 Total NPT content changes in 1 and 3 mM $Pb(NO_3)_2$ treated *B. napus* plants in comparison with control plants within 7 days of exposition

γ-glutamylcysteine synthetase, γ-ECS catalyzes the reaction of direct glutathione precursor production and therefore plays a key role in glutathione level regulation within the plant cell. Simultaneously serving as rate limiting enzyme of phytochelatin system's biosynthetic pathway γ-ECS was chosen to study phytochelatin system's enzymes response to lead stress. Higher in comparison with control plants γ-ECS activity was observed in both roots and shoots of *Brassica napus* plants stressed with 1 and 3 mM $Pb(NO_3)_2$ suggesting the enzyme stimulation by lead influence. The steady activity increase was noted in leaves for both lead concentrations up to two-fold higher value than that of control plants on the seventh day of exposition. In *Brassica napus* roots γ-ECS activity maintained a steady but still higher than control level in case of 1 mM $Pb(NO_3)_2$ and in case of 3 mM $Pb(NO_3)_2$ the two-fold activity jump was noted in the seventh day of exposition only. γ-ECS activity of root tissue was relatively higher for 3 mM $Pb(NO_3)_2$ and was thus stimulated proportionally to lead concentration applied (Fig. 4). Up to 7.7 times higher activity level of leaf tissue in comparison with root tissue is most probably a result of additional chloroplastidic enzyme isoform occurring in the shoots and missing in the roots.

The transcription activation mechanism involvement of γ-ECS stimulation as a result of lead stress was assayed by RT-PCR and confirmed quantitively by real-time PCR technique. For *Brassica napus* γ-ECS gene (*GSH1*) amplification a pair of primers designed basing on *GSH1* most conservative sequences from *B. juncea* and *A. thaliana* was used. In 1 mM $Pb(NO_3)_2$ treated *Brassica napus* roots γ-ECS mRNA accumulation was higher in comparison with control plants and transcription level increased regularly up from second day of exposition (data not shown). In case of leaf tissue transcription level did not significantly differ from controls throughout first 48 hours, the distinct mRNA accumulation drop in the fifth day of exposition and two-fold well correlated with enzyme activity changes increase in the seventh day were observed.

In both *Brassica napus* organs examined the increased γ-ECS mRNA accumulation in response to lead influence was observed. Similar γ-ECS transcript amount increase was noted by Schaffer *et al*., 1998 for Cu an Cd treated *Brassica juncea*. Good correlation between mRNA accumulation and γ-ECS activity fluctuations of lead stressed rapeseed plants confirms the transcription activation mechanism importance in phyto chelatin system's key enzyme regulation in response to lead stress.

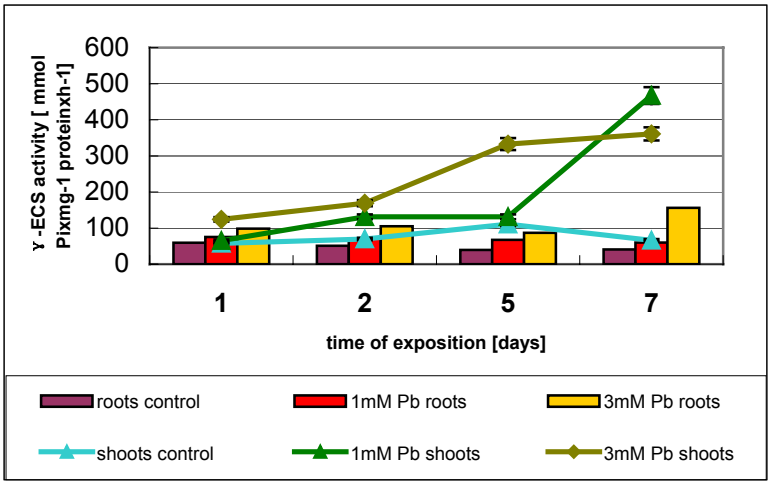
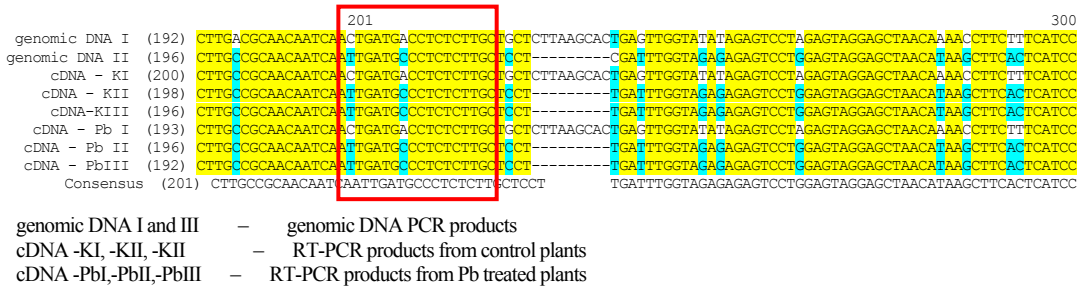


Fig. 4 γ-ECS activity changes dynamics in 1and 3 mM Pb $(NO_3)_2$ *B. napus* treated roots and shoots

*Brassica napus γ-ECS gene (*GSH1*) sequence, structural analysis and alleles occurrence*

Brassica napus γ-ECS gene was identified in one of John Innes Centre rapeseed genomic library clones using radioactively labelled probe. Sequence of the whole 8244 bp long insert was determined. The insert contained rapeseed *GSH1* promoter elements as well as reading frame (GenBank accession number AM265631) and approximately 2000 bp of sequence upstream from promoter region. Introns and 15 predicted exons layout was bioinformatically established basing on known sequence of homologous *GSH1* gene from *A. thaliana.* The sequence similar in 74% to *B. juncea* γ-ECS allel 1-1 promoter has been determined as one of putative promoters regions. Potential transcription initiation site was predicted using TSS P/Prediction of Plant Promoters software.

Various representatives of *Brassica napus* related plants show the diversity within *GSH1* gene. For example three alleles of the gene occur in *B. juncea* and *A. thaliana* is known for one, however transcribed into two different products. The predicted first exon area of lead treated and control *Brassica napus* DH O-120 plants genomic as well as cDNA obtained from total RNA was RT-PCR examined for diversity. Among both genomic DNA PCR products and cDNA PCR products two different sequence variants could be distinguished



genomic DNA I and III – genomic DNA PCR products
cDNA -KI, -KII, -KII – RT-PCR products from control plants
cDNA -PbI,-PbII,-PbIII – RT-PCR products from Pb treated plants

Fig. 5 Comparisons of two *GSH1*allels and their products from lead stressed and control *B. napus* plants

Variants differ with 9-nucleotide deletion/insertion within first 200 bp area of predicted first exon (Fig. 5). As 9 nucleotide deletion/insertion codes exactly 3 aminoacids (VLK) and does not interrupt or switch the reading frame the accidental character is unlike and indicates two distinct alleles of *Brassica napus* γ-ECS gene occurrence, both transcriptionally active in lead stressed and control plants. γ-ECS gene sequence from genomic library was compared with each variant to match one of them and it may be ascribed to *Brassica napus GSH1-1*. Further comparisons revealed the highest identity between rapeseed *GSH1-2* allel and *Brassica juncea* allel 1-2.

Conclusions

Regarding a relatively high lead tolerance and decent lead accumulation natural *Brassica napus* presents a promising phytoremediation prospect. The phytochelatin system of rapeseed plants responds distinctly to lead stress through quick non-protein thiols synthesis, γ-glutamylcysteine synthetase activity and mRNA accumulation increase. Therefore it is suggested that phytochelatin system participates to a significant extent in an overall *B. napus* defense towards heavy metals by their ions detoxification mechanism. At least two distinct γ-glutamylcysteine synthetase gene alleles occur in *Brassica napus* genome and both are trascripionally active in rapeseed plants under lead stress and without its influence. *Brassica napus* proved phytoremediative potential of lead, decent knowledge about phytochelatin system function and γ-glutamylcysteine synthetase genetics enable genetic engineering approach to obtain rapeseed plants highly useful in lead decontamination in soils.

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