# Phytoremediation Potential of Hemp (Cannabis sativa L.): Identification and Characterization of Heavy Metals Responsive Genes

**Article** *in* CLEAN - Soil Air Water · August 2015

DOI: 10.1002/clen.201500117



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**Research Paper**

# Phytoremediation potential of hemp (*Cannabis sativa* L.): Identification and characterization of heavy metals responsive genes[†]

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[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: [10.1002/clen.201500117]



**Received: 6 March 2015; Revised: 6 March 2015; Accepted: 15 June 2015**

**Abstract**

Soil pollution caused by heavy metals is one of the major problems throughout the world. To maintain a safe and healthy environment for human beings, there is a dire need to identify hyperaccumulator plants and the underlying genes involved in heavy metals stress tolerance and accumulation. The goal of this research is to explore the potential of hemp as a decontaminator of heavy metals by identifying the two important heavy metals responsive genes, *glutathione-disulfidereductase* (*GSR*) and *phospholipase D-α* (*PLDα*). The results revealed heavy metals accumulation; Cu (1530 mg $kg^{-1}$), Cd (151 mg $kg^{-1}$) and Ni (123 mg $kg^{-1}$) in hemp plants' leaves collected from the contaminated site. This shows the ability of the hemp plant to tolerate heavy metals, perhaps due to the presence of stress tolerance genes. In this study, partial sequences of putative *GSR* (215 bp) and *PLDα* (517 bp) genes were identified, responsive to heavy metals stress in hemp leaves. Both genes exhibited 40--60% sequence identity to previously reported genes from other plant species. Glutathione binding residues and conserved arginine residues were found identical in a putative *GSR* gene to those of other plant species, while the phospholipids binding domain and catalytic domain were found in the *PLDα* gene. These results will help to improve our understanding about the phytoremediation potential of hemp as well as in manipulating *GSR* and *PLDα* genes in breeding programs to produce transgenic heavy metals tolerant varieties.

**Keywords:** Gene characterization, Gene identification, Hyperaccumulator, *Glutathione reductase* gene, *Phospholipase* gene

**Abbreviations**: **GSR**, glutathione-disulfide reductase; **PA**, phosphatidic acid; **PIP5K**, phosphatidylinositol-4-phosphate 5-kinase; **PLDα**, phospholipase D-α; **ROS**, reactive oxygen species; **RT-PCR**, reverse transcriptase-polymerase chain reaction

## 1 Introduction

Soil contamination is increasing at an alarming rate due to a number of human activities such as release of industrial effluents, municipal wastes and waste sludge enriched with heavy metals that contaminate the surrounding environment [1--3]. Once the heavy metals contaminate the environment, they will remain a potential threat to humans and animals for many years [4]. Biological decontamination methods are considered safe for removing these pollutants, particularly from water and soil. In this regard, the phytoremediation technology is an environment friendly and cost-effective technology [5]. Many plant species have the ability to grow on contaminated sites and some of them are able to accumulate high concentrations of heavy metals in their tissues. To promote phytoremediation, it is necessary to find hyperaccumulater plants that have the ability to grow fast and accumulate high concentrations of metals [6]. Currently, more than 400 species of metal hyperaccumulator plants have been reported in the literature [7]. However, their low roots and shoots biomass

strongly limits their real application in this soil decontamination approach. An ideal plant for phytoremediation should have high biomass, a high tolerance to heavy metals stress and a high metals accumulating capability [8]. Hemp (*Cannabis sativa* L.) is an annual herb used in many types of non-food industries e.g. it provides raw material for natural fiber production [9]. There are certain characteristics of hemp, which make it very suitable for phytoremediation such as high biomass, long roots and a short life cycle of 180 days. In addition, hemp has a very high capability to absorb and accumulate heavy metals like lead, nickel, cadmium, zinc and chromium [9--11].

Identification of the functional genes or proteins that are involved in response to heavy metals stress is a fundamental step in understanding the molecular mechanisms of stress responses. A variety of reactive oxygen species (ROS) are produced in plants under biotic and abiotic stress conditions. ROS cause severe oxidative damage to biological molecules, DNA, proteins and lipids. Cells and tissues protect themselves from oxidative damage through up-regulation of a wide variety of antioxidant products [12, 13]. Among antioxidants, glutathione-disulfide reductase (GSR) is an abundant metabolite in plants with its diverse and important functions in the ascorbate glutathione cycle and signal transduction [14, 15]. Recent research has documented a regulatory role for glutathione in influencing the expression of many genes which are important in plants' responses to both biotic and abiotic stresses [16]. GSR enzyme activity has been studied in response to different environmental stresses such as salinity, heavy metals and it protects cells from oxidative damage [16]. Another class of antioxidant enzymes is the phospholipases. Phospholipases are key enzymes that catalyze the hydrolysis of structural phospholipids to form its product phosphatidic acid (PA) [17]. PA acts as a secondary messenger that regulates different protein like kinases, small G proteins, and PIP5K [18--22]. The *phospholipase D-α* (*PLD*) gene and its product PA are involved not only in stress signaling, but also in plant developmental signaling. The *PLD* gene has been suggested to regulate specific developmental and stress responses [23, 24]. So far, multiple forms of phospholipases such as D, C, and A have been characterized in plants. These enzymes are involved in cellular regulation, lipid metabolism, and membrane remodeling [25]. *PLD* genes play an important role in the signaling and production of hormones during different stress responses in plants [25, 26]. The transcription activity of both *PLD* and *GSR* genes increases upon exposure to various stresses, such as cold, drought and salinity [27--30].

The purpose of the present research is to identify the potential of hemp plants to decontaminate heavy metals polluted soils. This was done first by summarizing the already published data. Secondly, we identified and characterized *GSR* and *PLDα* genes from hemp plants. The hemp plant genome is still not sequenced and the genes responsible for heavy metals stress tolerance are unknown. Therefore, the identification, isolation and characterization of *GSR* and *PLDα* genes may allow us to deduce molecular pathways involved in metals tolerance and accumulation in the hemp plant.

## 2 Materials and methods

### 2.1 Samples collection and heavy metals determination

Hemp plant samples were collected from a metals contaminated site near Kohi Noor Textile mills in Rawalpindi,

Pakistan. The metals discharged from this industry include Pb, Zn, Cu, Co, Ni, Cr and Cd. Leaves of hemp plants were harvested and immediately collected in liquid nitrogen and preserved at --80 °C until further processing. For heavy metals determination, plant leaves were rinsed with distilled water and dried to a constant weight in an oven at 70 °C. The dried samples were ground, using an agate pestle mortar and kept in polythene bags. 2 g of prepared soil sample was digested with 15 mL nitric acid, 20 mL perchloric acid and 15 mL hydrofluoric acid and heated for 3h. After cooling, the digest was filtered into 100 mL flasks and diluted with distilled water. Likewise, dry powdered leaf samples were digested with 60% $HClO_4$, concentrated $HNO_3$ and $H_2SO_4$. The digested samples were analyzed for heavy metals (As, Cd, Co, Cu, Fe, Ni, Pb and Zn) using atomic absorption spectrophotometry in the COMSATS Institute of Information Technology, Abbottabad, Pakistan. The instrument settings and operational conditions were done in accordance with the manufacturers' specifications.

**2.2 Nucleic acids (RNA) isolation**

Total RNA extraction was carried out using the RNA prep plant pure RNA extraction kit (TIANGEN Biotech, Korea). To remove co-extracted DNA, the RNA samples were treated with DNaseI (TIANGEN Biotech, Korea). Total RNA concentration was determined by spectrophotometry at $A$ = 260 nm, Eq. (1):

$$\text{Total RNA} = \text{OD260} \times 40\ \text{ng}/\mu\text{L} \times \text{dilution factor} \quad (1)$$

The quality of total RNA was checked by running RNA samples on 1.5% agarose gel.

**2.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)**

To perform RT-PCR, cDNA was prepared from 2 µg of DNaseI-treated RNA samples, using oligo (dT) primer and reverse transcriptase enzyme (Topscript cDNA synthesis kit, Enzynomics, Seoul). RT reactions were incubated at 37 °C for 60 min and then heated at 94 °C for 10 min. Fragments of *GSR* and *PLDα* genes were PCR-amplified from 50 to 100 ng of cDNA equivalent, using degenerated primers. For the amplification of the *PLDα* gene, forward primer 5'-CACCATGATGATTTTCATCAGCC-3' and reverse primer, 5'-TATCATCAACAATCAT-3' and for the *GSR* gene forward primer 5'-GGAGCATCTTATGGAGGTGAAC-3' and reverse primer 5'-CAGTTTTTTCTTGTCGCCCAG-3' were used. All oligonucleotide primers were obtained from Generay Biotech (Shanghai). The total volume used for PCR reaction was 20 µL, containing 50 ng cDNA, 5 pmol of each pair of primers and 10 µL of 2X Master Mix (*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers). The thermocycler "Master cycler gradient" (Applied Biosystem) was used for amplification of the genes of interest. The reaction mixture was subjected to the following amplification program: Preliminary denaturation (94°C, 5 min), followed by 35 cycles of amplification (denaturation; 94°C, 30 s), hybridization 60°C, 30 s), elongation (72°C, 0.45 s) and final elongation at 72°C for 10 min.

**2.4 Analysis of PCR products (amplicons)**

The DNA fragments amplified by PCR were visualized on 1.5% agarose (Tiangen Biotech, Shanghai) gel electrophoresis in TAE buffer (0.04 M Tris, 0.001 M EDTA, pH 8) (Gendepot, USA), containing 0.60 µg $mL^{-1}$ ethidium bromide. A DNA ladder of 100bp-3kb (Enzynomics, Seoul, Korea) was used to compare the fragment sizes.

**2.5 Sequence analysis**

The PCR product obtained was sent to Laragen sequencing and genotyping (Virginia, CA). Nucleotide sequence

identity was determined using the online BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) while multiple sequence alignments were constructed using the online ClustalW2 program.

## 3 Results and Discussion

### 3.1 Hemp potential as a hyperaccumulator

The discovery of hemp's immense high potential in soil restoration began in 1998 in Ukraine's Institute of Bast Crops, where hemps were exclusively planted for the purpose of removing contaminants near the Chernobyl site. A number of studies conducted on hemp (*Cannabis sativa* L.) revealed that it can accumulate a considerable amount of heavy metals from contaminated soils due to its high biomass and deep roots, making it a good candidate for soil remediation. A hyperaccumulator plant is one that absorbs toxins, such as heavy metals, to a greater concentration than that the soil in which it is growing [31]. The results showed a higher accumulation of Cu (1530 mg $kg^{-1}$), Cd (151 mg $kg^{-1}$) and Ni (123 mg $kg^{-1}$) in hemp leaves collected from heavy metals contaminated sites, whereas all other heavy metals concentration remained negligible (Table 1). Therefore, the results suggest that hemp plants could be used for the remediation of Cu, Cd and Ni contaminated soils. Many studies have reported the accumulation of a variety of heavy metals such as Ni, Pb, Cd, Zn and Cr in hemp (Table 1), makes it suitable to be considered in soil remediation processes. In addition, a number of different studies have shown that heavy metals accumulation in hemp plants increases with the increasing concentration of metals in the growing solution/soil. However, the metal distribution in various parts of hemp is different. It has been proved that a higher concentration of metals in soils increases the heavy metals transfer from roots to hemp leaves and shoots. Moreover, accumulation tendencies of metals like cadmium, copper, and zinc in hemp are very similar to each other [32]. With the highest concentration of Cu, greater accumulation in both shoots and roots of hemp was observed. Similarly, when hemp was treated with 150 mg/kg Cu, a two- and eightfold increase in the Cu concentration could be observed in shoots and roots, respectively, as compared to the control [33]. Pb, Zn and Cd phytoextraction potential was studied in hemp with and without the addition of a chelate (10 mmol/kg EDDS). Pb, Zn and Cd concentrations in the biomass of *Cannabis sativa* were recorded as 1053 ± 125, 211 ± 16 and 5.4 ± 0.8 mg/kg, respectively. It was calculated that the ratios were 105, 2.3 and 31.7 times higher for Pb, Zn, and Cd, respectively, than in the control experiments. Similarly, Shi and Cai studied the Cd accumulation and tolerance in eight potential energy crops and they found that hemp is the best Cd accumulator and is considered an excellent candidate for phytoremediation [34]. In the same way, Linger and his colleagues conducted experiments to study the growth and photosynthesis ability of hemp under different cadmium concentrations. They observed that hemp could maintain its growth as well as the photosynthetic machinery, and long-term acclimation to constant Cd stress [35]. Shi and Cai observed that hemp has a strong tolerance for high Zn concentrations and showed small inhibitions in plant growth and photosynthetic activities [36]. In another study, hemp was grown and tested in two different soils, S1 and S2, containing 27, 74, 126 and 82, 115, 139 μg $g^{-1}$ of Cd, Ni and Cr, respectively. The results revealed that the mean shoot Cd content was 14 and 66 μg $g^{-1}$ for S1 and S2 soil, respectively, and the Ni and Cr uptake were limited. It has been suggested that hemp has the ability to avoid cell damage by activating different molecular mechanisms such as the antioxidant system [37].

### 3.2 Identification and structural features of the *GSR* gene, a putative heavy metal responsive gene of hemp

Degenerated primers were designed from the coding region after the alignment of *GSR* related genomes of different plants. These primer sequences were used to amplify partial cDNA sequences encoding putative *GSR*

gene from hemp leaves. For the identification of the *GSR* gene, total RNA was extracted from two different hemp plants and cDNA was synthesized from 2 µg of total RNA from each sample. After PCR, the amplified PCR product corresponding to the *GSR* gene from the two different hemp plants' leaves was analyzed on agarose gel electrophoresis and a clear band of 215 bp was detected in each sample as shown in lane 1 (hemp plant 1) and lane 2 (hemp plant 2) in Fig. 1. Amplified PCR products of the *GSR* gene from two hemp plants' leaves were sequenced and a partial sequence of 215 bp was received and identified as a putative *GSR* gene after the BLAST search. Characterization of the *GSR* gene was done with the already identified *GSR* genes from different plants. Schematic representation of target *GSR* included three motifs common to plant *GSR* representing three glutathione binding sites, the NADPH binding site and the redox-active disulfite bridge as shown in Fig. 2. Already published data showed a regulatory role of *GSR* in influencing the expression of many genes important in plants' responses to both biotic and abiotic stresses (Table 2). According to a recent study, heavy metals like Zn, Cu, and Cd essentially increased the activity of *GSR* involved in the ascorbate-glutathione cycle in sunflower [38]. In another study, *GSR* activity increased by 111 and 200% after seven and 14 days, respectively, in roots of wheat treated with Cd [39]. *GSR* also showed higher activity under Pb and NaCl toxicity [40]. Luo and his colleagues also reported the up-regulation of the *GSR* gene in perennial ryegrass under Cd stress [41]. Thus, up-regulation of *GSR* occurs as a defense mechanism against Cd, Cu, Zn and Pb stresses. Therefore, in this study amplified *GSR* product from 50 ng cDNA showed higher expression that could be due to heavy metals accumulation in hemp leaves (Fig. 2).

**3.3 Identification and characterization of the *PLDα* gene, a putative heavy metals responsive gene of hemp**

The *PLDα* gene was amplified by using degenerated primers designed manually from *PLDα* genome of different plants aligned by using the multiple alignment program.The amplified PCR product from two different hemp plants cDNA corresponding to *PLDα* gene was analyzed on agarose gel electrophoresis and a clear band of 517 bp was detected as shown in lane 1 (hemp plant 1) and lane 2 (hemp plant 2) in Fig. 3. The amplified *PLDα* gene was sequenced and then the sequence was analyzed by using bioinformatics tools. The characterization of the *PLDα* gene was done by multiple alignments of already published *PLDα* gene sequences (Fig. 4). Bioinformatics analysis showed that the $Ca^{2+}$/phospholipids binding domain, motifs 1 and 2 (catalytic domain) and conserved evolution boxes A, B and C were found identical to all other plant sequences reported previously. The *PLDα* gene was amplified from 50 ng cDNA and a higher expression of the *PLDα* gene could be observed as shown in Fig. 3. Higher expression of *PLDα* gene in this study could be due to heavy metals stress. Previous studies suggested that low concentrations of heavy metals stimulate *PLDα* signaling pathways which in turn lead to the production of ROS hence subsequent cell death accelerated by caspase-like proteases [42]. The *PLDα* gene has shown to be involved in ABA responses [43] which play an important role in numerous plant stress responses like cold, drought and salinity. In another study, a wheat putative *PLD*-encoding gene showed enhanced gene expression and thus contributed to the increase in *PLD* activity under copper stress [44]. Dai and his co-workers observed *PLDb1* mRNA in maize crop in response to Cd stress [45]. In addition, *PLDα* gene activity increased in different plants under abiotic stresses (Table 3) [27--29, 46]. This clearly showed the role of *PLDα* genes in abiotic stresses. However, to confirm *PLDα* genes' role in stress conditions, further studies, particularly functional studies will be required.

**4 Concluding Remarks**

The present research was conducted to evaluate the phytoremediation potential of the hemp plant and to identify the genes involved in heavy metals stress tolerance. The results as well as already published data demonstrated that hemp has a great potential to remove heavy metals, particularly Cu, Cd and Ni from contaminated soils and could be used in phytoremediation technology. PLDα and GSR are major antioxidant enzymes that protect plant cells against oxidative damage caused by ROS produced under stress conditions. The identification of *PLDα* and *GSR* genes may provide us with the basic understanding of mechanisms of heavy metals accumulation and tolerance in plants. The high expression of *PLDα* and *GSR* genes in hemp plant leaves indicates that these two genes express under heavy metals stress in order to help the plant cope with stressful conditions. The experimental approach used here can prove as a useful tool to characterize *PLDα* and *GSR* genes and their regulation at the transcriptional level. However, more experiments need to be conducted in the future to validate the results of transcriptional and post-transcriptional changes of *PLDα* and *GSR* genes under different heavy metals concentrations. Moreover, the studies regarding biological activities of *GSR* and *PLDα* genes will also be required to make the detailed elucidation of the physiological functions of these enzymes that play role in heavy metals stress tolerance. Further studies may lead to the production of heavy metals tolerant varieties of crop plants through genetic manipulation of the *PLDα* and *GSR* genes.

**Acknowledgements**

This study was financially supported by Higher Education Commission (HEC) of Pakistan through the Startup Research Grant project.

The authors have declared no conflict of interest.

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**Table 1.** Accumulation of heavy metals in different parts of Hemp plant in mg/kg

| Heavy metal | Leaves | Roots | Shoots | Seeds | Reference |
|---|---|---|---|---|---|
| Cd | 3.94<br>2.96 | | | 1.03<br>1.19 | [10] |
| | | | | 1.3--4.0 | [47] |
| | | >1000 | | | [48] |
| | 50-100 | 800 | 50-100 | | [35] |
| | 18.1 ± 8.0(S1)<br>58.8 ± 26.9 ( S2) | 109.2 ± 65.1 (S1)<br>1368.2 ± 1106.8 (S2) | 14 (S1)<br>66 (S2) | | [37] |
| | 151 ± 19 | | | | This study |
| Pb | 23.20<br>21.65 | | | 1.98<br>1.69 | [10] |
| | 39 ± 5 | | | | This study |
| Cu | | | | 10--12 | [49] |
| | 1148.16 ± 159.73 | 0.93 ± 0.08 | 44.41 ± 3.38 | | [33] |
| | 1530 ± 19 | | | | This study |
| Zn | | | | 42--57 | [49] |
| | | | | 42--94 | |
| | 4.5 ± 1.2 | | | | This study |
| Ni | | | | 1.6--6.1 | [49] |
| | 7.1 ± 1.4 (S1)<br>31.4 ± 8.1 ( S2) | 35.8 ± 18.9(S1 )<br>321.8 ± 241.6 (S2) | | | [37] |
| | 63.83<br>63.46 | | | 33.24<br>4.79 | [10] |
| | 123 ± 13.2 | | | | This study |
| Cr | | | | 598--877 | [49] |
| | 1.4 ± 0.8 (S1)<br>1.2 ± 0.8 (S2 ) | 6.2 ± 4.2 (S1)<br>9.0 ± 9.9 (S2) | | | [37] |
| | 25.3 ± 1.75 | | | | This study |

**Table 2.** *GSR* gene or protein in response to heavy metals in different plants

| Heavy metal | Plant species | Technique | Measurement | Reference |
|---|---|---|---|---|
| Zn, Cr, Cu | *Helianthus annuus* | RT-PCR, spectrophotometry | *GSR* gene expression and enzyme activity | [50] |
| Cd | *Triticum aestivum* | RT-PCR, Western-blot analysis | *GSR* gene expression and Protein quantification | [51] |
| Cd | *Lolium perenne* | RT-qPCR | *GSR* gene expression | [41] |
| Cd | *Populus tremula* | Glutathione reductase (GR) assay | GSR enzyme activity | [52] |
| Zn | *Spinacia oleracea* | Affinity chromatography | GSRenzyme activity | [53] |
| Cd, Cr | *Nicotiana langsdorffii* | RT-PCR | *GSR* gene expression | [54] |
| Cd | *Brassica juncea* | Glutathione reductase (GR) assay | GSR enzyme activity | [55] |
| Cu, Cd | *Arabidopsis thaliana* | Nothern blotting | *GSR* genes expression | [56] |
| Cd, Cu | *Helianthus annuus* | Glutathione reductase assay | GSR enzyme activity | [57] |
| Pb | *Lathyrus sativus* | Real time RT-PCR | *GSR* gene expression | [58] |

**Table 3.** *PLDα* gene in response to abiotic stresses in different crop plants

| Stress | Plant species | Technique | Measurement | Reference |
|---|---|---|---|---|
| Cu | *Triticum durum* | RT-PCR, Western blotting | *PLD* gene expression and enzyme activity | [44] |
| Cd | *Brassica juncea* | Northern blot analysis | *PLD* Gene expression | [59] |
| Pb | *Lathyrus sativus* | Real time RT-PCR | *PLD* Gene expression | [58] |
| Cd | *Zea mays* | Northern blotting | *PLD* Gene expression | [45] |
| Freezing | *Arabidopsis thaliana* | Northern blotting | *PLD* Gene expression | [60] |
| Drought | *Arabidopsis thaliana* | Immunoblotting | PLD protein | [61] |
| Salinity | *Solanum lycopersicon* | Western blotting | PLD Protein | [46] |
| Drought | *Zea mays* | PLD activity assay | PLD protein | [62] |
| Cold stress | *Chorispora bungeana* | RT-PCR | *PLD* gene expression | [27] |
| Drought | *Arabidopsis thaliana* | RT–PCR | *PLD* gene expression | [63] |
| Drought | *Arabidopsis thaliana* | Immunoblotting | PLD protein | [29] |
| Salt | *Glycine max* | qRT-PCR | *PLD* gene expression | [28] |

**Figure 1.** RT-PCR amplified *GSR* gene from two different hemp plants. DNA fragments of *GSR* gene were amplified from cDNA of two hemp plants and separated using 1.5% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. M: 100 bp DNA ladder marker (Enzynomics, Seoul, Korea), lane 1 (hemp plant 1) and lane 2 (hemp plant 2): 215 bp *GSR* gene in two different hemp leaves.

**Figure 2.** Schematic representation of target *GSR* gene products. Regions spanning between the vertical arrows correspond to the deduced proteins from hemp cDNA sequences isolated in the current study.

**Figure 3.** RT-PCR amplified *PLDα* gene from hemp leaves. DNA fragments of *PLDα* gene were separated using 1.5% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. M: 100 bp DNA ladder marker (Enzynomics, Seoul, Korea), lane 1 (hemp plant 1) and lane 2 (hemp plant 2): 517 bp amplified *PLDα* gene from two different hemp leaves.

**Figure 4.** Schematic representation of target *PLDα* gene products. Regions spanning between the vertical arrows correspond to the deduced proteins from hemp cDNA sequences isolated in the current study.

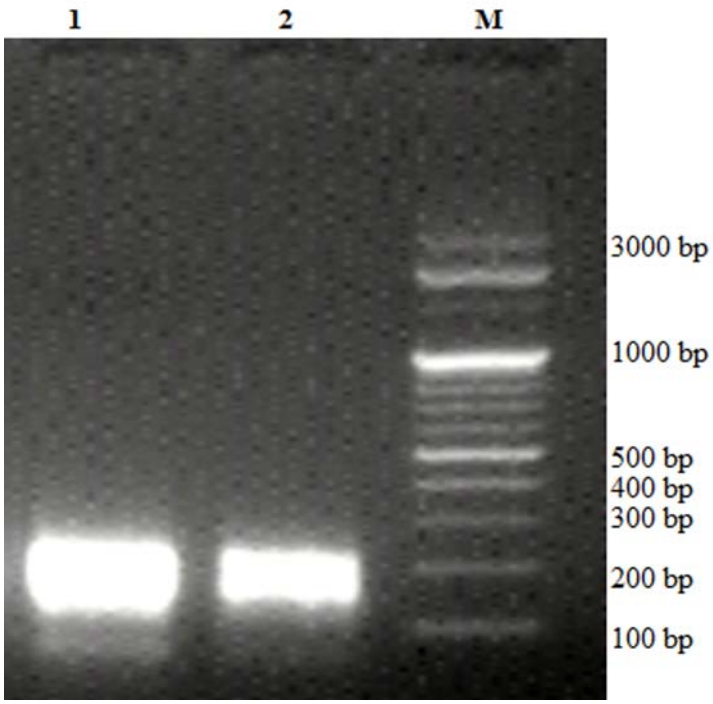
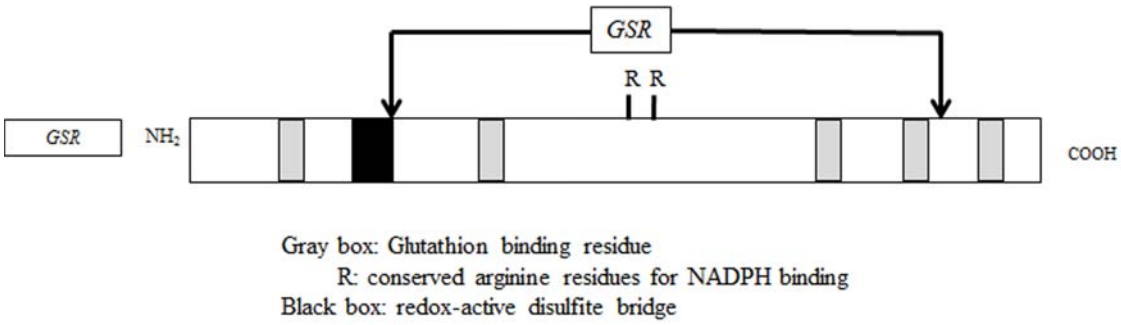



**Figure 1**




Gray box: Glutathion binding residue
R: conserved arginine residues for NADPH binding
Black box: redox-active disulfite bridge

**Figure 2**

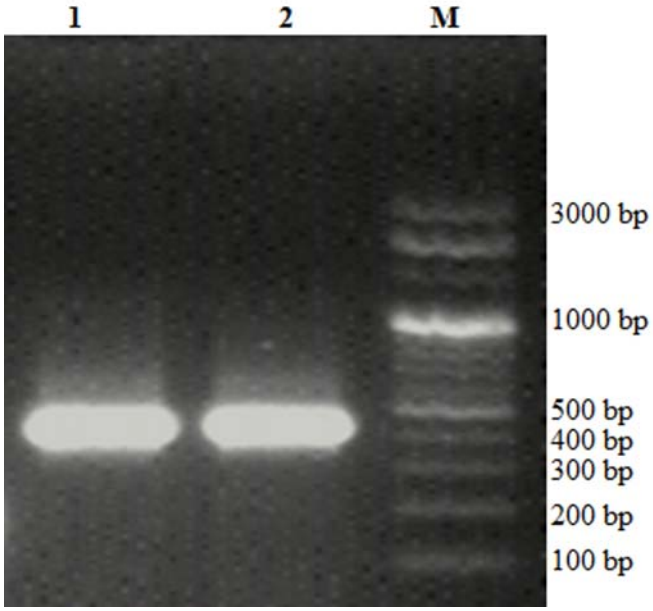
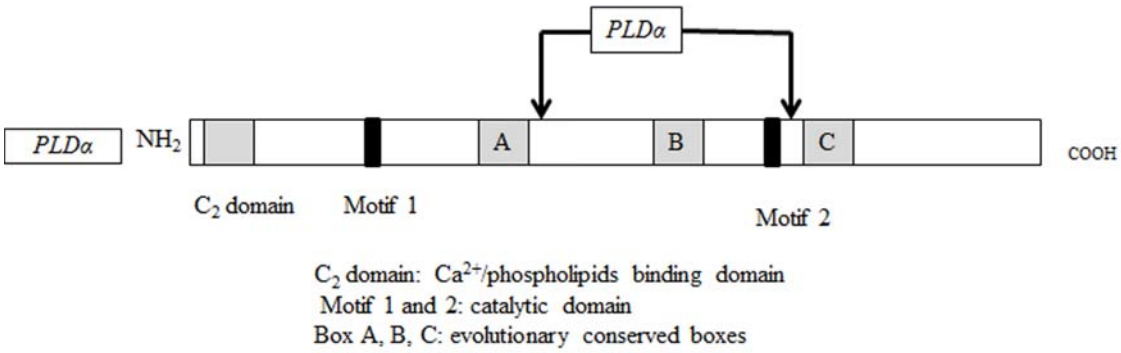



**Figure 3**




$C_2$ domain: $Ca^{2+}$/phospholipids binding domain
Motif 1 and 2: catalytic domain
Box A, B, C: evolutionary conserved boxes

**Figure 4**