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REGULAR ARTICLE

EXPRESSION OF PATHOGENESIS-RELATED PROTEIN GENES AND CHANGES OF SUPEROXIDE DISMUTASE ACTIVITY INDUCED BY TOXIC ELEMENTS IN *LUPINUS LUTEUS* L.

Klaudia Jomová[1], Melánia Feszterová[1], Martin Morovič[2]*

Address: [1]Constantine the Philosopher University in Nitra, Faculty of Natural Sciences, Department of Chemistry, Tr. A. Hlinku 1, 949 74 Nitra, Slovak Republic,
[2]Constantine the Philosopher University in Nitra, Faculty of Natural Sciences, Department of Zoology and Anthropology, Tr. A. Hlinku 1, 949 74 Nitra, Slovak Republic

*Corresponding author: kjomova@ukf.sk

ABSTRACT

Plants exposed to oxidative stress conditions increase the activity of enzymes involved in antioxidant defence reactions and induce the gene expression of defence protein genes. In our experiments, the effect of toxic elements (Pb, Cd, As) on the activity of superoxide dismutase (SOD) and the expression of pathogenesis related (PR) protein genes have been investigated in *Lupinus luteus* L. root tips. The results have shown increased activity of three SOD isoenzymes classified as a Mn-SOD (I), Cu/Zn-SOD (II) and Fe-SOD (III). Moreover, a new isoenzyme form of Mn-SOD was observed following the application of high doses of Cd and As. The expression of the PR10 genes was monitored with gene-specific primers. Increased SOD activity as well as transcriptional activity of PR10 genes suggests their participation in defence reactions under the conditions of oxidative stress.

Keywords: Pb, Cd, As, SOD, PR-10 proteins, oxidative stress, RT-PCR, *Lupinus luteus* L.

INTRODUCTION

Agricultural, industrial and human activities lead to the accumulation of heavy metals and the elements with only partial metal properties (arsenic) in soil. These elements are subsequently absorbed and accumulated in different parts of a plant (**Volmannová *et al.*, 2008; Tóth *et al.*, 2010)** and act as stress factors. They are involved in ROS-generating mechanisms leading to the initiation of defence reactions in plants. Recent studies investigating the response of plants to the presence of toxic elements have indicated the changes in activity of antioxidant enzymes such as catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), **(Pereira *et al.*, 2002)**. SODs catalyse the dismutation of superoxide into H_2O_2 and constitute the first line of defence against ROS (Reactive Oxygen Species), **(Jomová, Valko, 2011; Valko, 2005; Alscher, 2002)**.

In plants, heavy metal accumulation is associated with the changes in gene expression, resulting in the de novo synthesis of specific proteins **(Sarowar *et al.*, 2005).** The majority of inducible plant proteins are pathogenesis related (PR) proteins that are synthesized in response to biotic or abiotic stress stimuli. The proteins of PR10 class have been shown to be ubiquitous in the plant kingdom **(Walter *et al.*, 1990)**. These proteins have been classified as ribonuclease-like proteins due to the structural similarity to ginseng ribonuclease **(Moiseyev *et al.*, 1997)**, but they also share similarity to pollen allergens **(Pinto et al., 2005)**. In *Lupinus luteus*, three subclasses of PR-10 proteins (LlPR10.1, LlPR10.2 and LlCSBP) have been identified **(Handschuh *et al.*, 2007)**. The studies have showed that *pr-10* genes are implicated not only in plant defence mechanisms, but constitute a substantial part of the plant developmental program. However, the exact biological function of these proteins remains still unclear. Heavy metal induction of PR-10 proteins reported in *Lupinus luteus* root tips indicates their possible role in defence reactions against heavy metals (**Przymusiński *et al.*, 2004).**

The aim of our study was to investigate the changes of SOD activity and PR-10 protein gene expression in response to increasing concentrations of toxic elements (Pb, Cd, As) in *Lupinus luteus* L.

MATERIALS AND METHODS

Plant material and germination

Seeds of yellow lupine (*Lupinus luteus* L.) were obtained from breeding station Hodowli Roślin Wiatrovo (Poland) and germinated according to the procedure of **Przymusiński, Gwóźdź (1999).** Toxic elements were applied in the form of solutions of $Pb(NO_3)_2$, $Cd(NO_3)_2$ and As_2O_3 with the following ion concentrations: 100, 300, 400, 500 $mg.dm^{-3}$ of Pb^{2+}, 50, 100, 200, 300 $mg.dm^{-3}$of Cd^{2+} and 50, 75, 100, 150 $mg.dm^{-3}$ of As^{3+}.

Assay of SOD activity

Enzyme extract was prepared from the root tips (**Pereira *et al.*, 2002)** and the total protein content was determined by the method of **Bradford (1976**). The SOD activity was determined on native PAGE gels (**Beauchamp and Fridovich, 1971**; modified by **Azevedo *et al.*, 1998**). Bovine Cu/Zn-SOD (Sigma-Aldrich,USA) was used as a control. SOD isoenzymes were classified separately following the pre-treatment of the gels in H_2O_2 and KCN before SOD staining (**Azevedo *et al.*, 1998**).

Gene expression analysis by semiquantitative RT-PCR

Total mRNA was extracted from the root tips using plant RNA Mini Kit (Quiagen, Germany). The 5.0 µl of total RNA (0.7 µg) was reverse-transcribed using 1.0 µl of oligo(dT) and 2 µl of RTenzyme mix (Phusion^{TM}RT-PCR kit, Finnzymes, Finland) in the thermal cycler (Mastercycler ep384, Eppendorf, Germany). Semiquantitative RT-PCR analyses were carried out with gene-specific primer pairs complementary to stress pathogenesis related (PR) protein genes and reference ribosomal protein (RP) genes (Tab.1).

Table 1 Sequences of gene-specific primers used for semiquantitative RT-PCR

Protein	Gene	Sequences of primer pairs	NCBI accession number	Gene product (bp)
RPS14	LlRPS14	F: GCCACTGGTGGAAACAAAAC R: AAACCAGTTCCAGCCACAAA	AF026079	193
RPL30	LlRPL30	F AATGGTTGCAGCCAAGAAAA R CCAACCTTTGCCAACATAGC	AJ223316	210
PR10.1c	LlPR10.1C	F: TTAAGGAAATGGAGGACCA R: ATCCACCATCAGGACCAGAA	AF180941	204
PR10.2a	LlPR10.2A	F ATCATCCCAAAGGCTGTTGA R GTCTGGCAATCCAACTCCAC	AF170091	192

Legend: RP –ribosomal protein (S14, L30), PR - Pathogenesis Related protein (10.2a, 10.1c), Ll – *Lupinus luteus,* LlRP(14/30) - reference genes, LlPR(10.2A/10.1C) – stress genes, F/R – Forward/Reverse (primer)

The sequences of all primer pairs were acquired from the EMBL nucleotide sequence database (http://www.ncbi.nlm.nih.gov) for *Lupinus luteus* L. and designed using Primer3 software (http://primer3.sourceforge.net/). In each PCR reaction, a 3 μl aliquot of the cDNA reaction mix, 0.5 μmol of each primer and 0.02 U/μl DNA polymerase (Phusion™ Hot Start DNA Polymerase, Finnzymes, Finland) were used in a total volume of 50 μl. PCR was performed according to the following protocol: 30s at 98°C an initial denaturation step followed by 35 cycles of 98°C for 10s, 60°C for 10s, 72°C for 40s. The final extension period was 5 min at 72°C. The PCR products were electrophoresed on a 1.5% agarose gel (Consort Pharmacia Biotech), and visualized by ethidiumbromide staining at 254 nm (Eastman Kodak Company, USA). The band intensity was quantified using the Kodak Imaging Software 4.0. The product size was estimated by comparing with the Low Molecular Weight (LMW) DNA Ladder® (New England BioLabs, UK).

RESULTS AND DISCUSSION

Toxic elements increase SOD activity

The extracts from root tips of seedlings exposed to toxic elements were used for SOD activity staining following PAGE. This technique is considered to be more efficient than the spectrophotometric method due to the removing of interference coming from non-SOD substances in the crude extract **(Chen, Pan, 1996).** The isoenzyme forms have been identified

according to the inhibition patterns to KCN and hydrogen peroxide (data not shown). Based on these patterns, SOD Ia and Ib were classified as a Mn-SOD (resistant to both inhibitors) and SOD II as a Cu/Zn-SOD (inhibited by both inhibitors). The signal detected in the presence of KCN indicated the presence of Fe-SOD (III). Our results have shown the increased SOD activity after exposure to solutions of all toxic elements (Fig 1A-C). Significant changes were observed mainly in the activity of Cu/Zn-SOD and Fe-SOD (II a III). The highest concentrations of Cd and As lead to the synthesis of a new isoform of Mn-SOD.

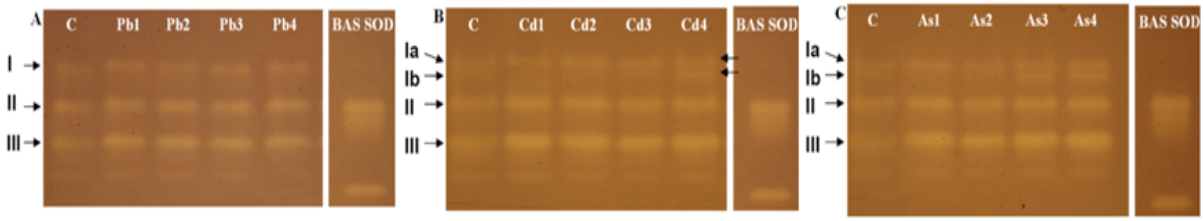

Figure 1 SOD activity staining following PAGE in root tips extract of *L. luteus* treated with Pb^{2+} ions (A), Cd^{2+} ions (B), As^{3+} ions (C). C – control (water) variant, Pb(1-4) – 100, 300, 400, 500 mg.dm^{-3} of Pb^{2+}, Cd(1-4) – 50, 100, 200, 300 mg.dm^{-3} of Cd^{2+}, As(1-4) – 50, 75, 100, 150 mg.dm^{-3} of As^{3+}, BAS SOD - Bovine activity standard SOD, Ia, Ib – Mn-SOD isoenzymes, II – Cu/Zn-SOD, III – Mn-SOD.

Many different SOD patterns observed with various metals have showed the variability according to the plant species and tissue **(Pereira *et al.*, 2003; Cardoso *et al.*, 2002).** It has been reported that changes in SOD activity as well as other antioxidant enzymes as a result of stress treatment do not have to correlate with the mRNA levels **(Donahue *et al.*, 1997)**.

Expression profiles of PR genes

To ascertain whether increased amounts of toxic elements (Pb, Cd, As) can affect the PR-10 gene expression, the expression of LlPR10.1C and LlPR10.2A genes was monitored by semiquantitative RT-PCR with gene-specific primers. The rRNA genes (LlRPL30 and LlRPS14) were used as reference genes to compare the mRNA transcription level. The results have shown an elevated transcriptional activity of genes for PR-10 proteins in all variants treated with toxic elements in comparison with the control (water) variant (Fig.2-4). The expression of PR-10 genes appears to be the appropriate tool for the oxidative stress detection and was described by several authors.

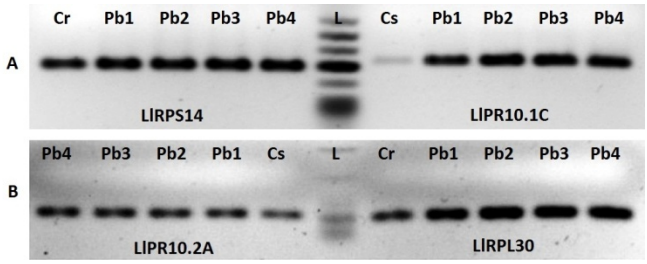

Figure 2 Agarose gels of PCR products of stress genes LlPR10.1C (A) and LlPR10.2A (B) after $Pb(NO_3)_2$ treatment. Cr – control (water) variant of reference gene, Cs – control (water) variant of stress gene, Pb(1-4) – 100, 300, 400, 500 mg.dm^{-3} of Pb^{2+}, L – LMW DNA ladder.

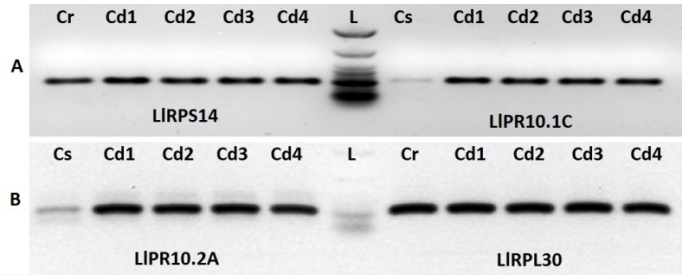

Figure 3 Agarose gels of PCR products of stress genes LlPR10.1C (A) and LlPR10.2A (B) after $Cd(NO_3)_2$treatment. Cr – control (water) variant of reference gene, Cs – control (water) variant of stress gene, Cd(1-4) – 50, 100, 200, 300 mg.dm^{-3} of Cd^{2+}, L – LMW DNA ladder.

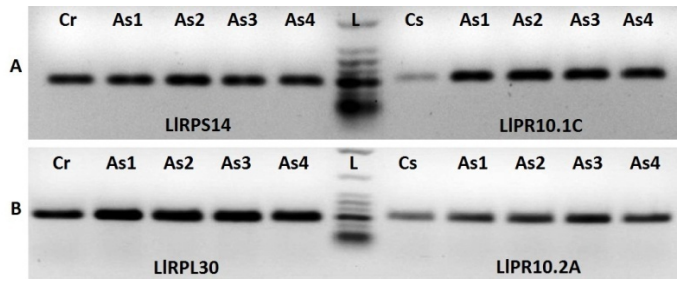

Fig 4 Agarose gels of PCR products of stress genes LlPR10.1C (A) and LlPR10.2A (B) after As_2O_3 treatment. Cr – control (water) variant of reference gene, Cs – control (water) variant of stress gene, As(1-4) – 50, 75, 100, 150 mg.dm^{-3} of As^{3+}, L – LMW DNA ladder.

Handschuh *et al.* (2007) have observed LlPR-10 gene expression in yellow lupines after treatment with hydrogen peroxide (H_2O_2) that immediately increases the ROS concentration in plant cells. **Przymusiński, Gwóźdź (1999)** have described the accumulation of small 16 kDa polypeptides in *L. luteus* root tips after treatment with Pb^{2+} ions. These proteins were identified as a member of PR-10 pathogenesis related proteins. Later, authors have proved the accumulation of these polypeptides in roots of *L. luteus* exposed to other stress factors such as metals (Cu, Cd, Zn), osmotic and salt stress as well as ethylene, salicylic acid, and hydrogen peroxide (**Przymusiński *et al.*, 2004**). These results suggest participation

of the PR proteins in plant defence to various stressors. The strong signal intensity of PCR products of reference genes in comparison to PCR products of stress genes in control water variants demonstrates their higher transcriptional activity in non-stress conditions. The results indicate that ribosomal protein genes are suitable for a comparison of mRNA transcription.

CONCLUSION

The increased SOD activity, as well as the expression of PR-protein genes induced by studied toxic elements, indicates that these molecules are regulated by oxidative stress. The ability of PR-protein genes to elevate their transcriptional activity at already low concentration of toxic elements makes them suitable markers for the detection of oxidative stress. The tested ribosomal protein genes ("housekeeping" genes) are useful to compare the transcriptional activity of inducible genes. Though the concentrations of the studied elements were higher when compared to those found in the natural environment it is important to establish which concentration induces the antioxidant defence providing the protection of plant against oxidative damage and find the plant species able to accumulate the high concentration of toxic elements.

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