Detection of HMA5, PCs and MT2 Genes Expression in *Vicia faba* Under Heavy Metal Stress Using Quantitative Real-Time PCR

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Abstract

In order to achieve phytoremediation during agricultural production, it is essential to identify high genotypes yield that is able of accumulating many types of heavy metals not in the edible parts which have significant nutritional value but in the non-edible parts. This study conducted to estimate the heavy metal ATPases 5 (*HMA5*), Phytochelatins synthase (*PCs*) and metallothionein 2 (*MT2*) genes expression in plant *Vicia faba* in response to an elevated concentration of copper and zinc in nutrient media. Using Quantitative Real-Time PCR (RT-qPCR) technique, the results of hydroponic culture methods with high concentrations of copper (75 µMol / L) and high concentration of zinc (500 µ Mol / L) showed high expression level for the three genes of *Vicia faba* plant compared with control. Established that the expression of the genes under the influence of copper ion was higher than the expression under zinc ion influence. Besides that, gene expression increased with increased exposure time to zinc ion, also in the case of copper ion exposure time, all genes expression slightly increases with increased exposure time. In response to excess copper and zinc, an increase in the expression of genes (*HMA5*, *PCs* and *MT2*) involved in plant protection, providing the possibility of its transfer from the cytosol to the apoplast demonstrate that this plant might be useful for phytoremediation of moderately polluted areas with copper or zinc.

Keywords: *Vicia faba, HMA5, PCs, MT2, copper, zinc, RT-qPCR.*

Introduction

Heavy metals can be a major problem for different organisms as may be reactive with several chemicals essential to biological processes [1]. Many chemical and physical methods were used for soil reclamation and remediation, however, these techniques usually required high maintenance costs and may lead to secondary pollution [2].

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Absorption and transportation are crucial mechanisms of plant tolerance to heavy metals which can be performed by heavy metal-associated isoprenylated protein (*HMA*) [3]. The phytochelatins (*PCs*) are also linked to metalloids with heavy metals to produce *PC*-metal complexes which are very stable and have less toxicity rather than free metal ions present in the cells. Under natural conditions, *PCs* are actively involved in the degradation of various glutathione conjugates in comparison to other metalloids[4]. Metallothioneins (*MTs*) bind to different metal ions due to high affinity of the sulfur molecule in the thiol group of cysteine and exert a major role in the detoxification of heavy metal stress[5].

In the Mediterranean Basin and Arab countries, Faba bean (*Vicia faba*) considers as one of the most important legume crops, which is an efficient nitrogen fixer [6]. Faba bean is a plant that had the ability to grow in various climatic zones [7]. Additionally, it can be consumed throughout the year, as it can be utilized in both raw and processed forms. For a human, it is mostly the seed grain that is consumed, whereas the pods are used as animal feed. the plant pods have micro and macro compunds, however, they can a good source of functional phytochemicals [8]. The nutritional importance of Faba bean is lying behind a prominent high protein [9] that offers a valuable amount of energy [10]. This legume plant has also therapeutic potentialsas it provides the precursor to a drug used in Parkinson's disease treatment [11]. Parts of faba bean plants and its processing products such as grains, hulls, and flowers considered asgood source of fiber and non-nutrient secondary metabolites which could be salutary to human health [12]. Many reports showed that high protein foods particularly animal-based have a high probability of causing intestinal problems in the long term of use, especially cancer, due to a lack in antioxidant compounds and an abundance in dangerous metabolites [13, 14]. Enhancements of the quantity and quality of food proteins could be done by a combination of legume plants such as faba bean with different plant-based foods [15]. The consumption of faba bean seeds provides some essential amino acids required for normal growth and repair of damaged tissues. Further research that can lead to a reduction in the current extent of yield variability is needed, thus faba bean may prove to be a key component of future arable cropping systems where declining supplies and high prices are likely to constrain the affordability and use of fertilizers [16]. As the development of food production is in continuously processes, and due to environmental and dietary beneficial of faba bean, it could be grown in the market within the next years, and become economical and valuable agricultural products such as soybean [17].

Recent studies indicated the ability of *Vicia faba* to tolerate the elevated concentration of heavy metals such as copper [18-20]. The metal translocation in plants mainly depends on plant species and type of metals [21]. Negative effect of oxidative stress may results either from the increasedconcentration of essential metals (micronutrients) like Zn, Cu and Ni or nonessentials such as Pb and Cd [22, 23]. Heavy metals are common soil pollutants. Therefore, the effectiveness of plant defense system (antioxidants) in plants should be crucial to clarify the mechanisms of plant tolerance to heavy metals. Production of tress concentration of different metabolites, such as amino acids (prolin, ascorbic acid and histidine) and peptide (e.g., glutathione GSH) or phytochelatins (PC), could be essential for the mechanisms of defense against heavy metals effects. These low molecular weight antioxidants detoxify oxygen free radicals. Also formation of metals non-toxic complexes by binding with nonprotein compound but rich in -SH groups wich is an important factor to perform plant's tolerance to heavy metals ions[24-27].

Based on the above and due to the importance of Faba bean in detoxification of heavy metal ions, this work conducted to investigate the expression of *HMA5*, *PCs* and *MT2* genes in plant *Vicia faba*.

Material and Methods

Treatment of seeds

Local Faba bean seeds were disinfected by washing thoroughly with tap water for 15 minutes, sterilized with 2 % v/v sodium hypochlorite (Clorox) for another 15 minutes and then washed extensively with sterilized distilled water.

Sowing and cultivation the initial stage of seedlings

Disinfected seeds were sown on the surface of moist perlite in plastic containers (40 cm x x 30 cm x 8 cm) with holes at the bottom, placing the container on a tray. To maintain the desired humidity the top of the container closed with a glass plate and removing it only after sprouting.

Seedlings replantation in hydroponic system

At the age of 14 days, the plants removed from the perlite by using a spatula without damaging the roots. Then the roots washed in a small volume of water to remove the perlite particles. The plants are then cultivated in 1-liter pot, at a rate of three plants per pot. Aeration and mixing of the nutrient solution carried out by a continuous and uniform supply of air. In water culture plants grown in a growth chamber at the temperature range of 23-25 / 18-20 C° (day/night), 16:8 h light: dark photoperiod [28]. For growing in hydroponic, an MS

medium was adopted [29, 30] and pH adjusted to 5.8.

Experimental conditions

In the experiments used 5 to 6-week old plants with 3-4 fully developed leaves. Exposure started by the introduction of $CuSO_4$ to the culture medium at concentrations of 75 µMol [18] and $ZnSO_4$ at a concentration of 500 µMol [31]. Changing the culture medium performed every 5 – 7 days. As a control, plants grown on standard MS medium.

Gene Expression Study

Gene expression for *HMA5*, *PCs* and *MT2* genes were determined using RT qPCR technique by comparative Ct values of specific amplification for each gene to measure the level of gene transcription (mRNA level) [32]. The Ct of 18srDNA was used as an endogenous control for calibrating the Ct values of other genes [33].

RNA Extraction

Total RNA was extracted from the 5gm of fresh leave tissue (after 1 and 2 weeks of exposure to heavy metals) with the use of TRIZOL® reagent (Invitrogen, USA) according to the manufacture's protocol. For lysis,1 ml of Trizol solution was added to each sample. For three phases separation, 0.2 ml of chloroform was adding then tubes centrifuge for 10 min at 12000 rpm. RNA samples then concentrated using isopropanol followed washing using 70% ethanol. Finally, RNA pellet diluted using nuclease-free water. Quantus Fluorometer (Promega, USA) was used to determine the concentration of extracted RNA. For 1 µl of RNA, 199 µl of diluted QuantyFlour dye was mixed. After 5 minutes of incubation at room temperature in a dark place, RNA concentration values were detected.

Quantitative Real-Time PCR (RT–qPCR)

All RT-qPCR studies were designed to comply with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines where applicable or practical. RT-qPCR reactions were carried out with a mic real-time PCR system using GoTaq® 1-Step RT-qPCR System (Promega). Each 10 µl reaction volume contained 1 µl of RNA, 5 µl (2X) GoTaq® 1-Step RT-qPCR, 3 µl dH2O, and 0.5 µl (10µM) of each primer. The sequences of the selected genes were found in the American National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) nucleotide sequence database. The selection of primers for the coding part of the target genes was carried out using the VectorNTI 9.0.0 program. The primer designed to amplify mRNA from:

HMA5, F: 5-GACAACGACGATTCTCTGAGTAA-3

R: 5-TAACACAAGCAGCACAAGTCAT-3,

PCS, F: 5-ATCAGACCACCATTGACGACTT-3

R: 5- GAACTCACAAGACGAGGAACATCT-3,

MT2,F:5-GTCTTGCTGTGGAGGGAAACTGT-3

R: 5- GGGTTGCACTTGCAGTCAGAT-3)

18s rRNA gene as endogenous control, F: 5-GAGTGATGTGCCAGACCTAGGAATT-3

R: 5- ATGCTGATCCGCGATTACTAGC-3.

The reaction conditions included cDNA synthesis step of 37ºC for 15 min. followed by an initial denaturation step of 95ºC for 30 s, 40 cycles of 95ºC/20 s, 60ºC/30 s and 72ºC/30 s. The dissociation curve was obtained by heating the amplicon from 65 to 95ºC. A non-template control was also included for each gene. The primer annealing temperature was calculated using the Vector NTI Suite 9 program [34].

Results

The genes expression at the transcription level was evaluated by estimating change in folding level of mRNA transcripts using the RT-qPCR technique (Table 1).

Table 1 The estimated values of HMA5, PCS and MT2 genes expression under the influence of copper (75 µ Mol. / L) and zinc (500 µ Mol. / L)

Time	Groups	Ct 18sRNA	Ct HAM5	Folding	Ct PCS	Folding	Ct MT2	Folding
1st week	Control	26.4	27.90	1.00	20.90	1.00	21.20	1.00
	CuSO4 75 µMol.	27.5	27.80	2.30	21.00	1.37	21.70	1.52
	ZnSO4 500 µMol.	26.2	27.00	1.62	20.10	1.04	21.00	1.07
2nd week	Control	27.5	29.30	1.00	20.90	1.00	22.20	1.00
	CuSO4 75 µMol.	28.2	28.50	2.83	21.40	1.68	22.20	1.62
	ZnSO4 500 µMol.	27.3	28.20	1.87	21.00	1.19	21.70	1.23

Folding = 2^{-DDCt}

DDCt = DCt (gene) – DCt (control),

DCt = (gene value from RT qPCR) – (18sRNA value from RT PCR),

The expression activity assessed at the level of the total content of individual transcripts at 7 and 14 days of growing plants in MS media with a high concentration of $CuSO_4$ and $ZnSO_4$ separately as compare with plants that grow without heavy metal stress as control.

Copper vs. zinc as stress type

Results shown in Fig.1 indicate that *HMA5* gene exhibited a constant activity, and its expression was significantly higher in response to copper than zinc ions. On the other hand, *PCs* and *MT2* gene exhibited a varied activity, and its expression was slightly higher under the influence of copper than zinc.

HMA5 vs. PCS and MT2 as the investigated genes

Results shown in Fig.1 indicate that *HMA5* gene exhibited a constant activity, and its expression was slightly higher than other two genes (*PCs* MT2) under influences of copper and zinc at the 1st week and the 2nd week of exposure.

1st vs 2nd week as expossure time.

HMA5 gene exhibited a constant expression enhanced with longer exposure to copper and zinc, that the expression level increased significantly with increased exposure time. While *PCs* and *MT2* genes showes increas in gene expression under influence of copper and zinc comparing with control but there were no significant diffrenece between the level of expression at different exposure time (Fig.1).

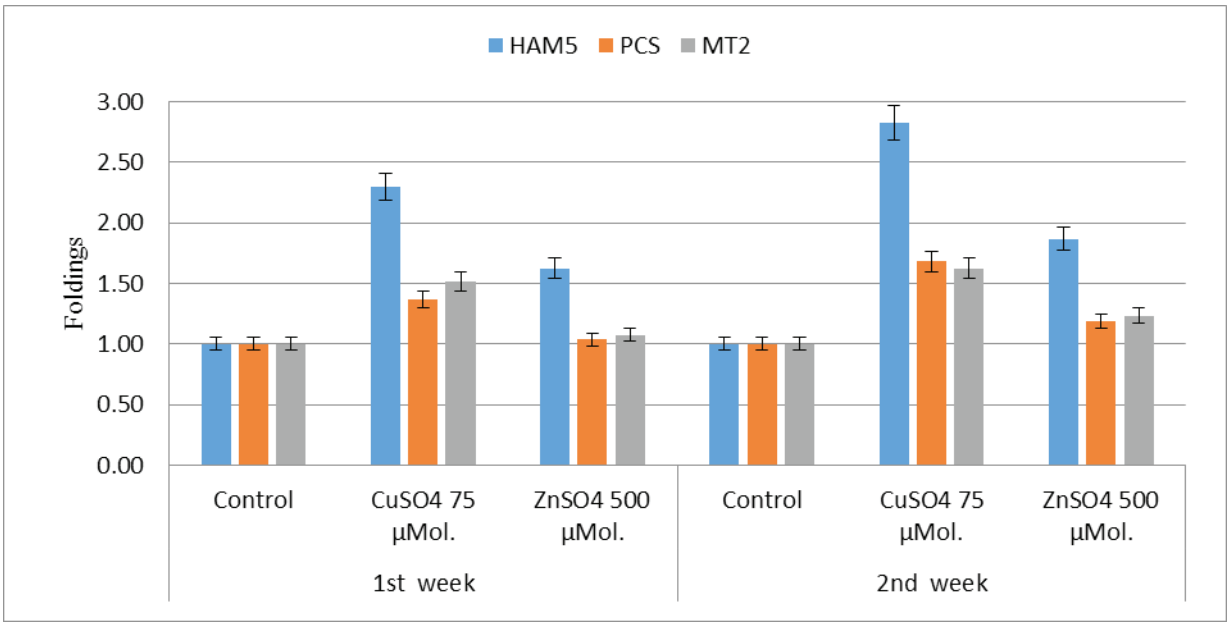

Fig. 1 HMA5, PCs and MT2 genes expressions under influence of copper (75 µ Mol. / L) and zinc (500 µ Mol. / L). (Standard error bars represent 5 % value)

Discussion

The resistance of *Vicia faba* plant to the toxic effect of copper and zinc ions could be related to changes in the expression of *HMA5*, *PCs* and *MT2* genes that involved in the regulation of intracellular homeostasis of the plant [35].

The obtained results showed that in all tested samples, the differences in the expression of the studied genes under the different heavy metals influence are mild. At the same time, manifested activity with an excess of heavy metals in the medium confirms their participation in the protective reactions of the investigated plants. This applies to the participation of membrane transporter gene *HMA5* [36] and the chelation of copper and zinc ions with the participation of phytochelatin synthase gene (*PCs*) [37, 38] and metallothioneins genes (*MT2*) carrying the excess copper and zinc ions from the cell to the extracellular space (apoplast) [39]. The activation of the expression of these genes encoding a chelation and membrane transporter, transferring excess copper or zinc from the cytosol into the cell wall, where its detoxification is carried out by binding with pectins and hemicellulose. Activation of these genes expression in leaves of *Vicia faba* plants may be one of the reasons for the increased resistance of *Vicia faba* to the toxic effect of excess copper or zinc in the medium [40]. This can serve to protect the plant from the toxic effect of increased content of $CuSO_4$ in the medium [41].

Conclusions

The increased activation of *HNA5*, *PCs* and *MT2* genes expression may indicate the formation of stress-protective mechanisms of plants from the toxic effect of high concentrations of copper ions in the environment.

Under the influence of excess copper or zinc, an increase in the expression of genes involved in plant protection was established, providing the possibility of its transfer from the cytosol to the apoplast *HMA5* which exhibit a higher expression level than *MT2* and *PCs* genes.

Vicia faba plant is potentially useful for phytoremediation of moderately polluted areas with copper or zinc ions.

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