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Differential expression of antioxidants, Fe and Zn transporter genes in wheat under Pb stress

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Abstract

Wheat (*Triticum aestivum* L.) is an important crop of the world and is considered as an essential food for one third of the world's population. Wheat yield and productivity are affected by many heavy metals including lead (Pb). In order to determine the genetic response of wheat against Pb stress, wheat was grown hydroponically with three different levels of Pb stress: 0, 75 and 225 µM. Total RNA was extracted from the leaves of two wheat cultivars 'Galaxy-13' and 'Punjab-11' in order to determine the expression of genes involved in detoxifying oxidative stress such as glutathione S-transferase (*GST*), glutathione reductase (*GR*), phospholipase Dα (*PLDα*) and zinc (Zn) transporter 8 precursor (*ZIP8*) and iron (Fe) regulatory transporter1 (*IRT1*). *In silico* characterization was performed to determine the sequence similarity of genes of this study in wheat and other crop plants. The results showed an increase in *GST*, *GR* and *PLDα* genes expression under severe stress conditions in both cultivars and the high expression of these genes could be important to protect cells from oxidative stress. Moreover, these genes could detoxify most of the secondary reactive oxygen species which are produced due to Pb stress. However, *ZIP8* gene expression was high under mild stress condition while *IRT1* gene expression was low in response to severe stress. In conclusion, high expression of *GST*, *GR* and *PLDα* genes could help wheat to survive under Pb stress and one or more of these genes could be used to screen Pb sensitive and resistant cultivars as well as in genetic transformation to develop Pb resistant wheat.

Key words: gene-expression, glutathione reductase, glutathione S-transferase, lead toxicity, phospholipase Dα, *Triticum aestivum*.

Introduction

Wheat (*Triticum aestivum* L.) belongs to Poaceae family, which is also called as Gramineae (Watson, 1990). This family consists of lots of cereal crops like rice, barley, rye and maize so making it the most important family among plant families. Over 600 million tons of wheat is produced annually. Maize, rice and wheat are the major crops produced and cultivated worldwide (van Ittersum et al., 2013). In West Asia, Europe and Africa, wheat has remained a staple food for the last ten thousand years. Wheat is the most widely grown crop in the world cultivated on approximately one sixth of the total arable land. It grows on a wide range of land and climatic conditions from Arctic Circle to high elevations near the equator. In Pakistan, 9 million hectares of wheat is grown during spring and winter seasons. About 80% of farmers grow wheat from December to January and these two months are considered to be the most suitable season for its growth.

Heavy metals and salts cause the environmental pollution which has become a severe problem all over the world. The mobilization through extraction of heavy metals from ores and other processing for various applications has led to the discharge of these elements into the surrounding environment (Abbasi et al., 2016). Continuous industrialization and disturbance of biogeochemical cycles cause the serious problem of heavy metal accumulation in the environment. Serious health and environmental problems have been observed due to the buildup of these metals in the soil and water (Ali et al., 2013). The heavy metals that cause environmental pollution include cadmium (Cd), copper (Cu), mercury (Hg), lead (Pb), arsenic (As) and zinc (Zn). The persistent deposition and subsequent accumulation of Pb occur in river sediments, soils and water (Stewart et al., 2015). The transformation and accumulation of Pb occur in plant body from soil through root uptake.

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In root, there is rhizosphere which facilitates the process (Lynch, Whipps, 1990), and Pb affects top fertile soil and down into the soil its concentration decreases.

Pb is considered as a slow acting poison which decreases the crop productivity (Gubrelay et al., 2015) and it is placed in the second number of all dangerous elements by the Agency for Toxic Substances and Disease Registry. Natural sources of Pb contamination are volcanic eruption and weathering (Chagué-Goff et al., 2013) and anthropogenesis activities including mining, smelting, combustion of fuel, synthetic fertilizer use, building construction, Pb-acid batteries (Mukai et al., 2001). A research conducted by Farooq et al. (2012) on the samples of soil and grass collected from 100 m distance from the edge of roads of most polluted cities of Pakistan found 125 to 87 mg kg^{-1} Pb, respectively in the soil and grass of highly populated cities of Pakistan, particularly Faisalabad and Lahore. Rice, maize, wheat and other major crops have been damaged due to Pb poisoning which has resulted in shrinking of root and shoot length, and stagnation of antioxidant activity (Fahr et al., 2013). Heavy metal toxicity occurs in plants as the accumulation of large amounts of heavy metals takes place inside the cells. Heavy metals can be divided into two groups: redox inactive (Cr, Fe, Co and Cu) and redox active (Zn, Ni, Cd, Pb and Al). Plant exposure to lethal levels of heavy metals activates extensive series of metabolic and physiological changes (Dubey, 2010). Yet, different heavy metals have different impact inside the plant; the whole toxic effect varies among different heavy metals. The recorded prevalent visual indication of heavy metal toxicity is a decrease in plant growth as well as necrosis, leaf chlorosis, lost turgor, seed germination reduction, and a disabled photosynthetic apparatus, often associated with progression of aging processes or death of plant (Bačkor et al., 2007). The above mentioned effects are linked to biochemical and molecular changes and overall structure. Heavy metals also decrease assimilation of carbon by inhibiting the enzymes involved in carbon dioxide fixation. In response to different heavy metals, a decrease in photosynthesis was observed with time and dose dependent manner (Gill, 2014).

A lot of research has been conducted by using heavy metals accumulator, hyper accumulator, heavy metal-tolerant, heavy metal-sensitive plant genotypes and transgenic approaches to find out the mechanism of heavy metal tolerance in plants (Wang et al., 2013; He et al., 2014; Fan et al., 2016). A large number of heavy metal-hyper accumulating and heavy metal-tolerant plants producing more glutathione enzymes can tolerate heavy metal stresses. For example, an increase in glutathione S-transferase biosynthesis plays a major role in nickel tolerance for nickel hyper accumulator. Moreover, *Sedum alfredii* known as a new Zn hyper accumulator and Pb accumulator exhibited a rise in glutathione content in response to Zn and Pb treatments. It is suggested that glutathione, rather than phytochelatins, is likely to be involved in transport of Pb and Zn, hyper-accumulation, accumulation and tolerance (Gill et al., 2013). Therefore, the published literature showed that Pb is considered as a toxic contaminant and decreases the yield of most crops. Therefore, in this work, we studied the expression of glutathione S-transferase (*GST*), glutathione reductase (*GR*), phospholipase Dα (*PLDα*), Zn transporter 8 precursor (*ZIP8*) and Fe regulatory transporter1 (*IRT1*) genes in wheat in order to ascertain their response to Pb stress conditions.

Materials and methods

Plant cultivation and lead (Pb) treatments. Seeds of two wheat (*Triticum aestivum* L.) cultivars 'Galaxy-13' and 'Punjab-11' were provided by Agriculture University Faisalabad, Pakistan. 'Galaxy-13' and 'Punjab-11' are both high yielding cultivars in Pakistan. This experiment was carried out in August, 2016 in Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan. One hundred seeds of each cultivar were soaked in distilled water for 24 hours and then 12 pots were filled with soil. Seeds from two wheat cultivars were entrenched in moist tissue layers and folded with foam from outside. Four sandwiches were prepared. Seeds were placed in each pot; out of which 6 pots with sand and soil substrate contained 'Glaxy-13' and 6 pots contained 'Punjab-11'. They were kept at 25°C in an incubator for one week. After that, they were transferred to hydroponics culture. The hydroponic arrangements were made in a greenhouse of COMSATS Institute of Information Technology, Abbottabad. After one week of germination, plants were transferred to the continuously aerated 250 mL bottles. Nutrients were given in one-fourth quantity at first day and it was raised to half and then full after every three days (Table 1). 75 and 225 µM of Pb nitrate Pb $(NO_3)_2$ as stress factor were given to the plants. One fourth of solution of 75 and 225 µM Pb was given at first day followed by half and then full concentration after every three days, respectively. Three replicates for each treatment in complete randomize design were performed in the greenhouse conditions.

Harvesting of plant leaves and total RNA extraction. Harvesting of plant leaves was done after one and a half week of cultivation in hydroponic culture. RNA was extracted from two wheat cultivars'Galaxy-13' and 'Punjab-11'. RNA extraction was done from leaves using reagent Wizol™ (Wizbio Solutions, Korea) according to the manufacturer's instructions. The size and quality of the RNA was determined by observing the bands. RNA quantification was done by a spectrophotometer NanoDrop (Thermo Fisher Scientific).

Table 1. The concentration of nutrients applied to wheat cultivars 'Glaxy-13'and 'Punjab-11' in hydroponic solution

Nutrients	Chemical used to provide nutrient	Molecular weight g	Concentration used in hydroponic solution
P	KH_2PO_4	136.09	0.1 mM
K	K_2SO_4	174.27	1.0 mM
Ca	$Ca(NO_3)_2$	236.15, 147.02	2.0 mM
Mg	$MgSO_4$	246.48	0.5 mM
Fe	Fe-EDTA	367.05	60 µM
B, Mn, Zn, Cu, Mo	H_3BO_3, $MnSO_4$, $ZnSO_4$, $CuSO_4$, $(NH_4)_2MO_7O_2$	61.83, 169, 287.54, 159.60, 1235.86	10 µM, 2 µM,0.5 µM, 0.2 µM,0.05 µM

Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR). cDNA was prepared by using the TOPscript™ cDNA Synthesis Kit (Enzynomics, Korea) by using 4 µg of total RNA according to manufacturer's instructions. Primers for selected genes were designed on the basis of their guanine-cytosine (GC) content and melting temperature by using software *Primer 3 plus* (Untergasser et al., 2012). Total volume used for PCR reaction was 20 µl; it contained 50 ng of cDNA, 1 µl of each pair of specific primers, 8 µl of 2X Master Mix (containing TaqDNA polymerase, dNTPs, $MgCl_2$ and reaction buffers) and 9 µl of double distilled water. The thermocycler Master cycler gradient (Sigma-Aldrich, USA) was used for amplification of genes of interest. The reaction mixture was submitted to an amplification programming in which the annealing temperature (Tm°) and elongation time (x) were adapted according to each couple of primers used. The amplification program was as follows: preliminary denaturation (95°C, 5 min), followed by 30 to 35 cycles of amplification, denaturation (95°C, 30 sec), hybridization (Tm°, x min), elongation (72°C, 30 sec) and a final elongation (72°C, 10 min). Annealing temperature of each selected gene is given in Table 2.

Table 2. List of specific primers for each gene used in this study

Gene	Primers	Sequence	Annealing temperature °C
Glutathione S-transferase	forward reverse	5'-CAGGCTAGATTCTGGGCTG-3' 5'-TGGAACAAGTGCTATATCCAC-3'	60.5
Glutathione reductase	forward reverse	5'-GGAGCATCTTATGGAGGTGAAC-3' 5'-CAGTTTTTTCTTGTCGCCCAG-3'	60
Phospholipase Dα	forward reverse	5'-CACCATGATGATTTTCATCAGCC-3' 5' -TATCATCAACAATCAT-3'	60
Fe regulatory transporter 1	forward reverse	5'-TCGCTCATGCTCAGCTTCTA-3' 5'-AAGAGCTGGTGGAAGCACAT-3'	55
Zn transporter 8 precursor	forward reverse	5'-ACCACAATCCCAAGCTCAAG-3' 5'-TCTTCCTCATCAAGGCGTTC-3'	57
Actin	forward reverse	5'-TGGTGTTAGTCACACGGTTC-3' 5'-CTGCAGAAGTGGTGAAAGTG-3'	54

Analysis of PCR products and construction of phylogenetic trees. The amplified PCR product of each gene was visualized by 1% agarose gel electrophoresis (Tiangen Biotech, China). A DNA ladder (Omega Bio-Tek) of 100 bp was used to compare the fragment sizes. For phylogenetic analysis, protein sequences of selected genes were taken in FASTA format using the accession numbers from National Center for Biotechnology Information (NCBI). These sequences were compared for identifying homologous sequences by using Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed by using robust phylogenetic analysis software at NCBI. Multiple alignment sequences were obtained using the program *Clustal Omega* with default settings and similarity of sequences was also calculated by using this software.

Results

Gene expression analysis of GST, GR and PLDα genes. The expression pattern of stress related genes *GSH*, *GR* and *PLDα* were analysed in two wheat cultivars 'Galaxy-13' and 'Punjab-11' under Pb stress. Results of expression analysis showed that there is fluctuation in the expression of *GST* gene in both cultivars. In 'Galaxy-13' there was a little increase under severe stress conditions, i.e. 225 µM as compared to control (C) while at moderate stress (75 µM) expression also increased but not that high as in severe stress. A high expression was shown by 'Punjab-11' under severe stress as compared to moderate stress and control (C). Expression study of *GR* showed enhanced overall expression of 'Punjab-11' and 'Galaxy-13' under severe Pb stress, i.e. 225 µM as compared to the control. While low expression was shown by moderate (75 µM) Pb stress. Results from expression analysis of *PLDα* gene was studied under severe (225 µM) and moderate (75 µM) Pb stresses. Both cultivars 'Galaxy-13' and 'Punjab-11' have shown high expression during severe stress as compared to both controls. At moderate stress, the expression was low (Fig. 1).

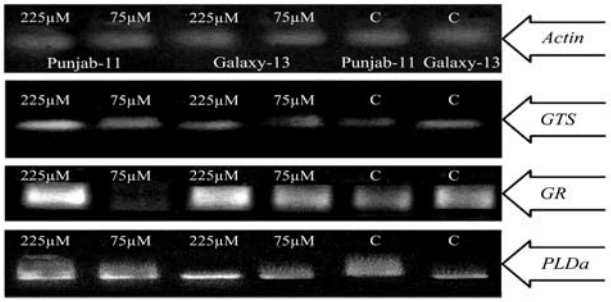

Figure 1. Expression of *GST*, *GR* and *PLDα* genes under various lead (Pb) stress conditions, i.e. 0 (control – C), 75 and 225 µM (actin was used as a reference gene)

Gene expression analysis of ZIP8 and IRT1 genes. Result from expression analysis of *ZIP8* showed a higher expression in moderate (75 µM) stress conditions in cultivars 'Galaxy-13' and 'Punjab-11'. While, surprisingly, there was a down regulation of this gene in severe (225 µM) stress as compared to both controls (Fig. 2). Expression analysis of *IRT1* showed no expression for moderate (75 µM) stress in 'Galaxy-13'. While low expression was shown in severe (225 µM) stress in 'Punjab-11' and 'Galaxy-13'. In the control of both cultivars *IRT1* gene was equally expressed.

Phylogenetic analysis. Highly similar sequences of *GST*, *GR*, *PLDα*, *ZIP8* genes of maize and other plants were selected for *in silico* characterization to construct phylogenetic trees. Multiple sequence alignment showed many conserved regions in gene sequences which determines their similarity and shows homology among them. This phylogenetic analysis showed that *GST* gene of Arabidopsis and *Hordeum* is originated from *Triticum aestivum*. Arabidopsis and *Hordeum GST* gene has the maximum similarity and the same function of this gene is supposed in Arabidopsis under Pb stress. However, *GR* showed the maximum similarity between *Sorghum bicolor* and *Oryza sativa* and both are originated from

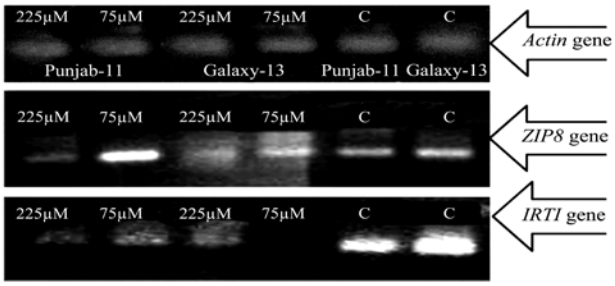

Figure 2. Expression of *ZIP8* and *IRT1* genes under various lead (Pb) stress conditions, i.e. 0 (control – C), 75 and 225 µM (actin was used as a reference gene)

Triticim urartu (Fig. 3). *PLDα* showed the least similarity with maize and maximum with *Aegilops tauschii* and *IRT1* had high similarity with *T. aestivum* and *O. sativa* and it is suggested that *IRT1* gene may show the same trend in *O. sativa* under Pb stress.

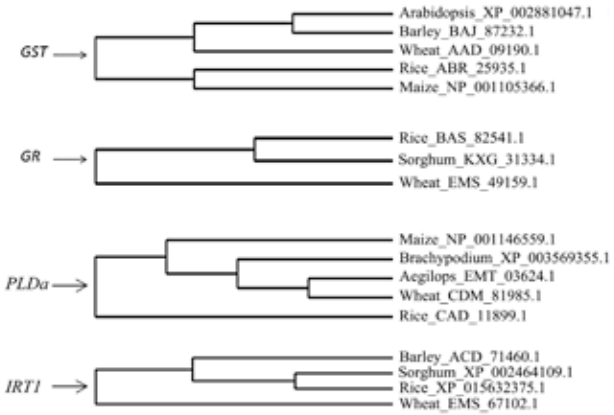

Figure 3. Phylogenetic analysis of *GST*, *GR*, *PLDα*, *ZIP8* and *IRT1* genes of wheat with the same genes in other plants

Discussion

Abiotic stresses, particularly heavy metal stresses reduce the yield of agricultural crops up to fifty percent (Gill, 2014). In response to Pb stress, wheat shows changes in biochemical pathways of important enzymes which in turn affect the expression of important genes (Singh et al., 2015). There are several researches that observed the effect of Pb stress on plants at transcriptional level (Herbette et al., 2006; Singh et al., 2015). In this research, we studied the expression of five specific genes in two cultivars of wheat under Pb stress. The result of this study indicated that there was an increase in expression level of *GST* in two wheat cultivars 'Punjab-11' and 'Galaxy-13' with high (225 µM) concentration of Pb as compared to the control and lower (75 µM) concentration. Higher expression of *GST* gene under high concentration of Pb may help plants to survive and enhance growth in the same way as higher expression of *GST* in transgenic tobacco increased plant growth and reduce stress impact (Roxas et al., 1997). In addition, it has been found that *GST* expression changes in wheat in response to Cd toxicity under mycorrhizal fungus association (Shahabivand et al., 2016). In the same way, an increase in *GST* was observed in response to nickel stress in both shoots and roots tissues of *T. aestivum* (Gajewska, Sklodowska, 2008).

Glutathione reductase enzyme is important for scavenging reactive oxygen species, which are unceasingly produced during abiotic stresses (Hossain et al., 2012). Glutathione reductase and glutathione play an important role in defining plant tolerance under different stresses (Rao, Reddy, 2008). Glutathione reductase activity was increased in the presence of Cd in *Arabidopsis thaliana*, *T. aestivum* and *B. juncea* (Khan et al., 2007; Mobin, Khan, 2007). Wu et al. (2015) showed that knock out of *GR* 3 increased the sensitivity of rice plant to salt stress. In our study, increase in *GR* expression was detected in the case of cultivar 'Punjab-11' at high concentration Pb treatment of 225 µM whereas under lower (75 µM) concentration, the expression was decreased. These findings are in accordance with the study of Hossain et al. (2012), where the decrease in *GR* was observed in mung beans seedlings in response to Cd stress, indicating that *GR* response differs greatly due to genotype differences and concentration of the stressor.

Phospholipases are important enzymes that catalyse the hydrolysis of structural phospholipids to phosphatidic acid (Gasulla et al., 2016). *PLDα* gene and its product phosphatidic acid are involved in stress signalling and also plant developmental signalling. Phospholipase D genes play a role in regulating stress and developmental responses (Hong et al., 2009). *PLDα* are involved in abscisic acid responses (Chen et al., 2016) which has a great role in various plants stress responses like drought, cold and salinity. Enhanced PLD-encoding gene expression was observed in wheat exposed to Cu stress (Navari-Izzoet al., 2006). High *PLDα* expression was observed in hemp under heavy metal stress (Ahmad et al., 2016). Furthermore, activity of *PLDα* gene in plants increases under abiotic stress (Bargmann et al., 2009). In our experiments, *PLD* expression was increased in cultivar 'Galaxy-13' under severe (225 µM) stress. It shows that Pb stress triggers the *PLD* gene expression and activity in wheat. In the same way, cultivar 'Punjab-11' showed high expression of *PLDα* in response to Pb stresses in both the treatments. This suggests the role of *PLDα* in both the wheat cultivars. Low (75 µM) expression in 'Galaxy-13' supports the previous studies of Yakimova et al. (2007) in which they showed that heavy metals at low concentration excite signalling pathways of *PLDα* due to which reactive oxygen species are produced that may cause cell death.

Gene of ZIP family is known to transport the Zn, Fe, Mn and Cd in several abiotic stresses (Mäser et al., 2001). In this study, a high expression of *ZIP8* is shown in the leaves of both wheat cultivars under moderate (75 µM) Pb stress while under higher Pb concentration, a decrease in expression was observed. A study of Tian et al. (2015) shows down regulation of *ZIP8* and *ZIP9* under Pb stress while an increase in *ZIP2* and *ZIP3* expression was observed in Louisiana iris. Low *ZIP* genes expressions was observed in rice such as *Oryza sativa* ZIP1 was strongly repressed by Cd, whereas *O. sativa ZIP3* was inhibited by calcium (Ramesh et al., 2003).

In our study, the expression of *IRT1* was low in the Pb treated wheat plants, whereas both controls were highly expressed. *IRT1* is a transporter of Fe while it also transports Mn and Zn in addition to Fe. It helps to accumulate Cd in Fe deficient plants (Zelazny, Vert, 2015). Further study is needed to know the role of *IRT1* in Pb accumulation.

The understanding of genetic mechanism is an important feature to develop plants having tolerance to different abiotic stresses and high yield. The tolerance of plants to heavy metals stress is regulated by the related network of biochemical and physiological mechanisms. Plants have both adaptive and constitutive mechanisms to survive in response to heavy metal stress (Viehweger, 2014). According to the data of our study, the over-

expression of *GST*, *GR* and *PLDα* under Pb stress could help a plant to cope with Pb stress. However, biochemical, physiological and molecular tactics are further investigated to find the fundamental mechanisms of tolerance, heavy metals accumulation and adaptive mechanisms to deal with stress of heavy metals. A few different mechanisms are originated by tolerant plants which consist of plasma membrane exclusion, immobilization, uptake and transport restriction, synthesis of specific heavy metal transporters, chelation and sequestration of heavy metals by particular ligands (phytochelatins and metallothiones), introduction of stress related proteins, polyamines biosynthesis, and signalling molecule like nitric oxide and salicylic acid (Viehweger, 2014).

Conclusion

Wheat productivity is negatively influenced by many heavy metal stresses like lead (Pb). In general, there are few studies conducted on the gene expression in wheat under Pb stress. In this research, we studied the expression of antioxidants genes glutathione reductase (*GR*), glutathione S-transferase (*GST*) and phospholipase (*PLDα*) that were over expressed in two wheat cultivars under high (225 µM) concentration and one or more of these genes could be used in genetic transformation to develop resistant wheat cultivars in the future. Moreover, this study provides the expression pattern of Pb responsive *GR*, *GST* and *PLDα* genes that could be used to screen sensitive and resistant wheat cultivars. However, differential expression of *ZIP8* and *IRT1* was observed in wheat cultivars under Pb stress and more research work on gene expression will be required to elucidate the function of these two genes in wheat. In addition, the proteomic study of *GR*, *GST* and *PLDα* proteins will be required to understand the full mechanism of these proteins under Pb stress.

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Antioksidacijos, Fe ir Zn pernašos baltymų genų raiška kviečiuose esant Pb stresui

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Santrauka

Kviečiai yra pasaulyje svarbūs žemės ūkio augalai, kurių grūdai yra laikomi trečdalio pasaulio gyventojų pagrindiniu maisto produktu. Kviečių derlingumą ir produktyvumą veikia daug sunkiųjų metalų, taip pat ir švinas (Pb).
Siekiant nustatyti paprastojo kviečio (*Triticum aestivum* L.) genetinį atsaką į Pb stresą, augalai auginti hidroponiškai, esant trims Pb streso lygiams: 0, 75 ir 225 µM. Siekiant nustatyti raišką oksidacinio streso detoksikacijoje dalyvaujančių genų: glutationo S-transferazės (*GST*), glutationo reduktazės (*GR*), fosfolipazės Dα (*PLDα*), cinko (Zn) pernešėjo 8 prekursoriaus (*ZIP8*) ir geležies (Fe) reguliavimo pernešėjo 1 (*IRT1*), bendra RNR buvo išskirta iš dviejų veislių 'Galaxy-13' ir 'Punjab-11' paprastojo kviečio lapų. Siekiant įvertinti kviečių ir kitų žemės ūkio augalų genų sekų panašumą, šios sekos buvo charakterizuotos *in silico*. Tyrimo rezultatai parodė, kad esant stipriam stresui *GST*, *GR* ir *PLDα* genų raiška padidėjo abiejų veislių kviečiuose, o aktyvi šių genų raiška gali būti svarbi ląsteles siekiant apsaugoti nuo oksidacinio streso. Be to, šie genai galėtų detoksikuoti daugumą antrinių reaktyviųjų deguonies rūšių, susidarančių dėl Pb streso. *ZIP8* geno raiška buvo didelė esant nedideliam stresui, o *IRT1* geno raiška buvo maža reaguojant į didelį stresą.
Apibendrinant galima teigti, kad aktyvi *GST*, *GR* ir *PLDα* genų raiška gali padėti kviečiams išgyventi esant Pb stresui, o vienas ar keli iš šių genų gali būti naudojami Pb jautrioms ir atsparioms veislėms nustatyti, taip pat genetinei transformacijai kuriant Pb atsparių veislių kviečius.

Reikšminiai žodžiai: fosfolipazė Dα, genų raiška, glutationo reduktazė, glutationo S-transferazė, švino toksiškumas, *Triticum aestivum*.