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UTILIZATION OF MOLECULAR MARKERS TO DETECT THE IMPACT OF HEAVY METALS ON PLANT GENOMES

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ABSTRACT

Heavy metal pollution is a worldwide problem affecting all living beings. The source of heavy metals in the environment can be natural such as volcanos and rock formation, however human activities, industrialization are the main sources of pollution. These activities have caused the reduction of plant diversity and have a negative effect on animal and human health. Plants are affected on multiple levels by these metals, they cause morphological and physiological changes, and they also have an impact on the molecular level. Changes on the molecular level due to abiotic stress are extensively studied. Scientists try to understand the mechanisms of stress response and the epigenetic mechanisms that lead to heavy metal stress resistance. This review article summarizes the molecular genetic methods available today and their ability to detect the changes in plant genome due to heavy metal stress. The article explores *omics* approaches such as genomics, different DNA marker methods such as RAPD, ISSR, IRAP as monitoring methods for heavy metal pollution in the environment, miRNA omics and transcriptomics as investigation methods for the interactions between genes and heavy metals.

Keywords: environmental stress, heavy metals, plants, omics, molecular markers

INTRODUCTION

Environmental pollution with heavy metals is a worldwide issue discussed by many branches of science. Globally, 10 million polluted sites were reported, of which more than half showed the presence of heavy metals **(He *et al*., 2015)**. Heavy metals have a devastating effect on ecosystems, human and animal health (Wu *et al*., 2016) (Figure 1). The toxicity of heavy metals is mainly because they are non-biodegradable and have a long half-life **(Mitra *et al*., 2022)**. Heavy metals can be defined as metals with density more than 5 $g.cm^{-3}$ **(Cevher-Keskin *et al*. 2019; Memon 2020)** and relative atomic mass greater than 20 **(Shakoor et al., 2013)** and according to **Nachana and Williams (2019)** above 40. More than half of the naturally occurring elements are heavy metals, some of these are essential micronutrients such as Fe, Mo, Mn and important trace elements like zinc (Zn), nickel (Ni), copper (Cu), cobalt (Co), chromium (Cr) **(Nachana and Williams, 2019)**. Lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd) are considered the most toxic, and are not known to have a role in living organisms **(Dutta *et al*., 2018, Nachana and Williams, 2019)**. Non-essential but also essential trace elements, when found in excess, cause morphological and physiological changes and even genetic mutations in living organisms **(Mitra and Chakraborty, 2022)**. The aim of the review is to summarize actual knowledge about the possibilities that provide different types of molecular markers in the research of the plant organism answering the abiotic stress caused by heavy metals.

HEAVY METALS IN THE ENVIRONMENT

Heavy metals are found naturally in the earth's crust, and in certain regions their concentration can be high **(Nachana and Williams, 2019)**. The natural ways in which heavy metals enter the environment include volcanoes, rock formation, weathering, and erosion of the soil. These events can expose nature to large amounts of Zn, aluminium (Al), Cu, Hg, manganese (Mn) and Ni **(Mishra *et al*., 2019)**. Human activities, however, are the greatest threat to nature regarding heavy metal pollution **(Mohammed *et al*., 2011)**. Industrialization has led to the release of toxic mixtures of heavy metals into the soil, water and air, and the effects are shown in the reduction of plant diversity, the negative impact on aquatic organisms, microorganisms, animals and on human health. As for industrial sectors, a large amount of heavy metals enters the environment through mining and processing **(Mohammed *et al*., 2019)**, further sectors like painting, tanning, textiles, dyes, papermaking, electroplating, leader industries **(Mishra et al., 2019)**, pharmaceutical industry **(Nachana and Williams., 2019)** production of batteries, thermometers, accumulators, laboratories, and cosmetic industry contributes to heavy metals pollution. Waste storage and landfills also represent a source of heavy metals contamination **(Mohammed *et al*., 2019)**. In urban areas traffic is a contributor to heavy metals pollution through emissions **(Sawidis *et al*., 2011)**. The emissions of heavy metals in Europe are decreasing; from 2005 to 2021, Cd emissions decreased by 30%, Hg and Pb emissions by more than 40% **(EEA, 2023)**. However, due to the longevity of heavy metals in the soil, the problems remain unsolved.

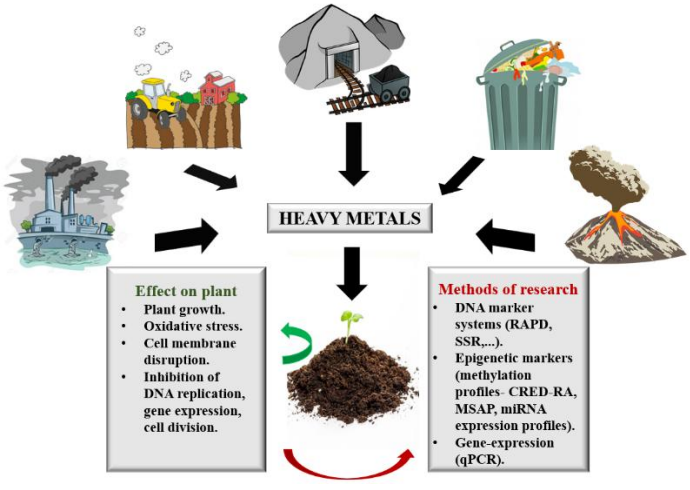

Figure 1 Depiction of sources of heavy metal pollution and toxicity effects on plants and the molecular genetics marker methodologies

HEAVY METAL TOXICITY TO PLANTS

Heavy metal toxicity is defined as the ability to cause adverse effects on organisms **(Rasmussen *et al*., 2000)**. Heavy metals have negative impact on plants from the morphological and physiological to the molecular level **(Gill *et al*., 2022; Angulo-Bejarano *et al*., 2021)**. At each of these levels, plants are affected differently. In addition to physiological and biochemical repercussions, stress response includes the inhibition of fundamental processes like DNA replication, gene expression, and cell division (Figure 2). This cascade of effects results in the impairment of plant growth **(Dutta *et al*., 2018)**. Heavy metals are a big problem with multiple risks, but there is an even bigger problem when interacting with other unresolving and growing problems, such as microplastic in the soil. The interaction of these two problems has a synergetic effect on multiple plants, reinforces the problem with heavy metals **(Ivy *et al*., 2023)**.

Moreover, the deeper study into the dynamics of plant stress responds to the role of epigenetic regulation assumes increasing prominence **(Ghosh and Roy, 2019)**. Accumulation of heavy metals in plants also depends on genotype **(Li *et al*., 2018)**. This fact is better summarized by **Wei *et al*., 2022**, the overall resistance of plant organisms to heavy metals is highly dependent on genotype. It has been found that adaptive responses to heavy metal-contaminated environments involve numerous physiological, molecular, genetic, and ecological processes that provide some species with the ability to survive in heavy metal-contaminated environments **(Sarma 2011)**. Major strategies include hyperaccumulating, tolerating, excluding, and chelating heavy metals **(Angulo-Bejarano *et al*., 2021)**. In addition to inherent mechanisms within plants that provide protection against heavy metal stress, targeted application of protectants can significantly enhance plant protection. One so useful is glycine betaine. Glycine betaine acts as a protective agent, bolstering the plant´s ability to cope with stress from heavy metals by mitigating oxidative damage and enhancing overall stress tolerance mechanisms **(Ali *et al*., 2020, Ahmad *et al*., 2020)**. Also, other protectants have been found to protect plants against heavy metals, such as phytohormones **(Bali *et al*., 2019)**, organic acids **(Sidhu *et al*., 2019)**, biochar **(Farooq *et al*., 2020)**, zinc oxide nanoparticles **(Ahmad *et al*., 2020)**, nanoscale sulfur **(Meselhy *et al*., 2021)**, methyl jasmonate (MJ) **(Mousavi *et al*., 2020)** and y-aminobutyric acid (GABA) **(Mahmud *et al*., 2017)**.

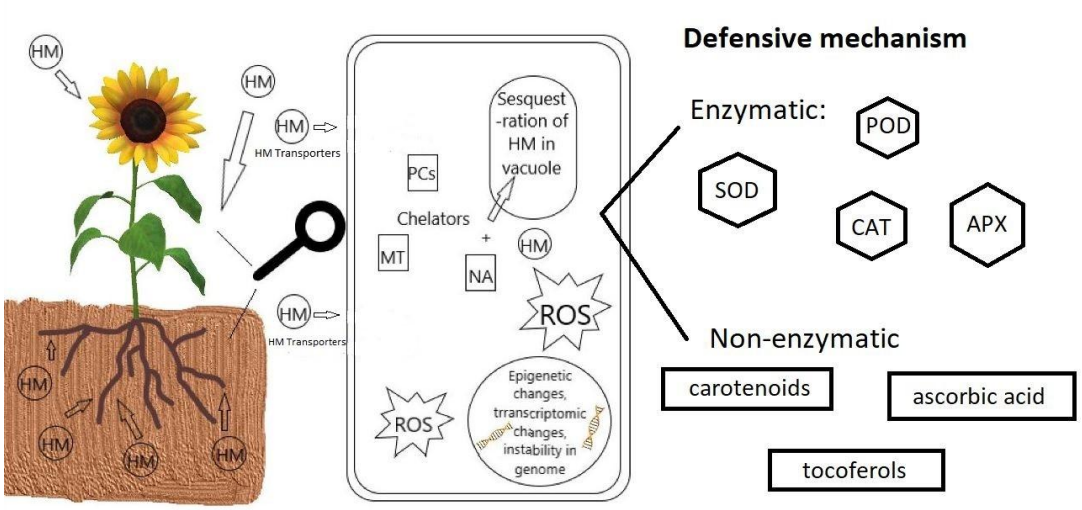

Figure 2 Plants' defensive mechanism after heavy metals exposure

The plant´s first line of defense against damage is the cell wall. When heavy metals breach this barrier, certain amount of them are immobilized by chelators **(Li *et al*., 2022)** include metallothioneins (MT) **(Zimmeri *et al*., 2005)**, phytochelatins (PCs) **(Kapoor *et al*., 2021)**, nicotianamine (NA) and plant defensins (PDF) **(Luo *et al*., 2019)** found in the cytoplasm. In plants, two key enzymes, ascorbate peroxidase (APX) and catalase (CAT), play a critical role in counteracting metal-induced hydrogen peroxide (H_2O_2) production. Furthermore, the different roles of APX and CAT highlight their different mechanisms for managing H_2O_2 levels in plants. While APX regulates the concentration of H_2O_2 and thus ensures precise signaling function, CAT efficiently scavengers excess H_2O_2 and thus prevents potential cell damage under heavy metal stress **(Cuypers *et al*., 2016)**. A decrease in CAT activity caused a lot of H_2O_2 to accumulate in the cell resulting in oxidative stress **(Pandey *et al*., 2009)**. In addition to enzymatic defense mechanisms, plants also use non-enzymatic systems for protection. These include compounds such as carotenoids, ascorbic acid, and tocopherol. Genotoxic stress causes genomic instability which ultimately results in plant growth inhibition. When plants are exposed to heavy metal stress, multiple signaling pathways are triggered. These lead to the activation of interrelated networks that combat oxidative damage. These include pathways involving calmodulin, mitogen-activated protein kinases (MAPK) and hormones. These signaling cascades work together to regulate the expression of specific stress-responsive genes that are critical for the plant´s adaptation to heavy metal stress **(Dutta *et al*., 2018)**. Hormones that are involved in plant protection processes against heavy metals include jasmonic acid (JA) and abscisid acid (ABA) **(Jan *et al*., 2023)**. Comparative analyses of heavy metals and their toxicity to plants have led to several conclusions. For *Cucumis sativa*, Cd, Cu, and Pb were studied. It was found that Cu had the most toxic effect and Pb seemed to be the least toxic. Authors attributed these results to the fact that the pH value of the soil, which was 4,3, played a major role **(An *et al*., 2000)**.

Heavy metals often trigger the DNA damage and lead to programmed cell death (PCD) process in plant cells. High concentrations of heavy metals may lead to necrosis or disturb the functioning of plant antioxidant mechanisms initiating PCD **(Bi *et al.,* 2009, Filippi *et al.,* 2019)**. Many studies indicate that reactive oxygen species (ROS) are responsible for activating the PCD process in cells in response to stress caused by heavy metals **(Petrov *et al.,* 2015, Kumar *et al.,* 2016, Filippi *et al.,* 2019, Sychta *et al.,* 2021)**. Although molecular mechanisms of PCD in plants, especially induced by abiotic stimuli, is poorly understood, it was demonstrated that plant proteases – caspase-like proteases (e.g., papain-like cysteine proteases, caspase-like proteases, metacaspases, the proteasome or vacuolar processing enzymes), play a crucial role in PCD regulation in plant cells **(Hatsugai *et al.,* 2005; van der Hoorn, 2008; Sychta *et al.,* 2020)**.

Cadmium

At the physiological and morphological level, Cd adversely impacts plant growth **(Shanmugaraj *et al*., 2019, Gautam *et al*., 2022)**. Furthermore, the reduction in Rubisco activity up to 74% at a Cd concentration of 50 µM, emphasizes inhibitory effect of Cd on the key enzyme in photosynthesis. This not only affects essential cellular processes but also emphasizes the connection between heavy metal stress, such as Cd, and crucial biochemical pathways within the plant system **(Dias *et al*., 2012)**. If plants are unable to prevent oxidative stress, cell membranes are disrupted. The activity of enzymes, especially peroxidase and CAT in cells, are involved in the prevention of Cd -induced oxidative stress **(Azevedo *et al*., 2007)**. This confirms the increased activity of malondialdehyde (MDA), peroxidase (POD) and CAT significantly in *Fagopyrum tataricum*. However, experiments on two different varieties showed differences. The longer the plants were under stress, the more MDA, POD and CAT activity was recorded **(Huo *et al*., 2023)**. The GSH reduction in *Lycopersicon esculentum* is directly proportional to the duration of Cd exposure to the plants. But on the other hand, the increase of PCs has been discovered **(Ammar *et al*., 2008)**. PCs, characterized by their structure of γ-Glu-Cys, serve as crucial components in plants' defense mechanism. The cysteine (Cys) component plays a main role in binding and sequestering heavy metals such Cd. After Cd exposure, PCs bind to the metal ions, forming stable complexes that are subsequently transported to the vacuole for storage. This sequestration process serves as a vital detoxification mechanism, effectively removing Cd from sensitive cellular area and preventing its detrimental effect in essential cellular processes **(Kapoor *et al*., 2021)**. But the stability of Cd-PCs complex is dependent on the pH in the cytosol **(Johanning and Strasdeit 1998)**. MT has a similar role in plants **(Zimeri et al., 2005)**. The whole process, when the plants are influenced by Cd has a detrimental impact on electron conductivity in *Pontederia cordata*, with a reduction of up to 87% observed at a concentration of 75 mg/l. This significant decrease in electron conductivity has effect, leading to noteworthy alteration in the permeability of leaf membrane **(Xin *et al*., 2020)**. Experiments on *Lycopersicon esculentum* have shown that increasing the Cd content in the soil (100 µM) also influences micro and macro elements, but the observed changes are not always

equal in each part of the plant. Decrease levels were observed for nitrogen, K and Fe in the leaves and conversely, increase occurred for Mg. As regards Cu, an increase was observed in the roots but decrease in the leaves. Mn content was reduced in roots but did not change in stems and leaves. However, at the concentration of 10 µM, the content in roots slightly increased **(López-Millán *et al*., 2009)**. At high concentrations (500 ppm), total water content and transpiration in *Brassica juncea* L. decrease, and conversely, there is an increase in proline **(Singh and Tewari, 2003)**. Several transcriptional factors and metal transporters have been demonstrated to be influenced by Cd exposure such as ABC-type transporters **(Brunetti *et al*., 2015)**, important for the process of vacuolar sequestration, natural resistance-associated macrophage protein (NRAMP), important for the membrane transport **(Zhang *et al*., 2022)**, heavy metal transporting-ATPase (HMA) and yellow stripe-like families (YSL) **(Tao and Lu, 2022)**.

Chromium

Cr toxicity in plants leads to oxidative stress, resulting in stunted growth, chlorosis induction, and wilting of leaves **(Sharma *et al*., 2020)**. At Cr concentration of 0,15 mM and 0,30 mM, the activity of APX and glutathione reductase (GR) increases. On the other hand, there was a decrease in the activity of dehydroascorabate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). These results have been observed on *Brassica juncea* **(Mahmud *et al*., 2017)**. Cr contamination in *Oryza sativa* affects H_2O_2, superoxide ($O_2{\cdot}-$), MDA and relative electrolyte leakage (REL) **(Yang *et al*., 2021)**. The MDA is a measure of lipid membrane damage from oxidative stress **(Adhikary *et al*., 2020)**. The increase of H_2O_2, $O_2{\cdot}-$, MDA and REL was present in both roots and leaves. But H_2O_2, O2- and MDA production was higher in leaves than in roots. REL was also higher in roots **(Yang *et al*., 2021)**. The carotenoid content of *Brassica parachinensis* L. initially tended to increase as consequence a low concentration of Cr, but with the increasing dose of Cr the content was decreased **(Kamran *et al*., 2021)**. Depending on concentration of Cr in *Cicer arietinum* plants the proline content increases, i. e. the higher concentration of Cr the higher proline content **(Singh *et al*., 2020)**. Also, Cr treatment has a negative impact on the intake of other vitals elements such as K, Mg, Zn, Ca and Fe **(Javed *et al*., 2021)**.

Mercury

Experiments on *Oryza sativa* indicate a different source of Cu penetrance to the parts of the plant. While the source of Hg in the roots is Hg found in the soil, up to 90% of the Hg in the above-ground part comes from the unpolluted atmosphere **(Tang *et al*., 2021)**. Tang and co-workers also point out that the amount of Hg^0 is also affected by high doses of CO_2. Elevated CO_2 concentration can reduce leaf stomatal conductance, limiting uptake of atmospheric Hg^0 into plant leaves. This phenomenon leads to a limitation process, indicating a complex relationship between elevated CO_2 levels and the ability of plants to take up atmospheric Hg. Elevated levels of Hg in plants result in alterations in cell membrane permeability. When plants are exposed to the inorganic form of Hg, it reduces the mitotic index in root tip cells and elevates the occurrence of chromosomal aberrations **(Patra and Sharma, 2000)**. The research highlights the dynamic character of antioxidant enzyme activity, specifically SOD and CAT, in *Medicago sativa* exposed to Hg. The observed changes in enzyme activity exhibited a significant correlation with the duration of exposure, highlighting the importance of the duration of Hg-induced stress on an antioxidant defense mechanism in the crop **(Zhou *et al*., 2008)**. Moreover, the reduction of potassium (K), magnesium (Mg) and calcium (Ca) in the leaves of *Triticum aestivum* L. following exposure to Hg form $HgCl_2$ underscore the impact of Hg on the plant´s nutrient dynamics (Sahu *et al*., 2012). Significant changes in plants begin to occur at 5 mg/kg of Hg. At low doses (1 mg/kg), the plant seems to show some changes. However, the plant seems to be able to cope with it, and its lifecycle isn´t radically disrupted **(Beauford *et al*., 1997)**. Some plants such as *Cyrtomium macrophyllum* are more tolerant to Hg and even this plant showed no signs of toxicity even after 500 and 1000 mg/kg of Hg in the soil. It is believed, that the plant´s resilience stems from a substantial increase in the levels of key antioxidants such as SOD, GSH and proline in leaves. This increase of protective compounds acts as a defense, effectively protecting the plant from potential damage caused by reactive oxygen species (ROS) **(Xun *et al*., 2017)**. Exposure to a concentration of 60 mg/l $HgCl_2$ has decreased the level of photosynthetic pigments (*chlorophyll a*, *chlorophyll b* and carotenoids) in *Zea mays* L. **(Abbasi *et al*., 2023)**.

Cobalt

Elevated levels of Co lead to several detrimental effects in plants. These effects include pale leaf discoloration, vein discoloration, and leaf loss, which can result in iron deficiency **(Hu *et al*., 2021)**. Additionally, Co toxicity has been linked to reduced growth parameters, including a decrease in leaf number, and hindered root growth **(Mahey *et al*., 2020)**. Furthermore, Co toxicity affects photosynthetic pigments, with reductions observed in both chlorophyll a and chlorophyll b levels and antioxidant enzymes, as reported by **Begovic *et al*. (2016)**. These negative Co-induced changes were also confirmed in *Phaseolus aures,* while except the changes in amount of chloroplast pigments, cobalt also affects activity of antioxidant enzymes (POD, SOD, APX, CAT) in the plant **(Tewari *et al*., 2002)**.

Lead

Pb toxicity exerts detrimental effects on plants across their developmental stages, from germination to crop maturation. Nevertheless, the degree of toxicity is influenced by exposure duration and Pb concentration. Elevated Pb levels disrupt the water and nutrient equilibrium within plants, leading to oxidative damage to their cellular components **(Zulfiqar *et al*., 2019)**. The impact of the Pb was investigating for three different plants simultaneously. A large decrease in root growth was observed in *Meditago Medicago sativa* and similar behaviour was also observed in *Triticum aestivum*, with the difference that no changes were observed at 50 µM. On the other hand, in *Zea mays*, root growth accelerated and decrease only at high (400 µM) Pb concentration **(Vasilachy-Mitoseru *et al*., 2023)**. One study shows that oxidative stress occurs in *Vicia faba* following Pb stress induction, primarily through the production of $O2{\cdot}-$ rather than H_2O_2. In addition, a correlation between oxidative stress and activation nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase in the plant was identified. Furthermore, the activation of NADPH oxidase appears to be associated with calmodulin and protein kinase. Research also investigated the mechanism by Pb entering the root of Vicia faba. It is very likely that Pb enters the root via a similar pathway like calcium ions. Pb causes DNA strand breaks and chromosomal changes. This genotoxic effect has been specifically observed in root tips in *Vicia faba*. It is important to mention that these negative effects occur due to ROS **(Pourrut *et al*., 2011)**.

Arsenic

In research conducted by **Coelho *et al*. (2020)**, it was demonstrated that toxicity arises from an excess of As in the forms of arsenite (As^{III}) and arsenate (As^{V}), leading to the overproduction of ROS and lipid peroxidation. These forms are also the most abundant and most available forms to plant **(Farooq *et al*., 2016)**. Experiments on *Zea mays* have shown that the most toxic form is arsenate followed by arsenite and demonstrable toxicity has also been shown for the dimethylarsinic acid (DMA) form, but this form represents a less toxic form than other two **(Abbas and Meharg, 2008)**. Through a series experiments conducted on transgenic and wild-type *Oryza sativa* plants, it was discovered that OsABCC1 plays a crucial role in plant detoxification of As Its location in the tonoplast of *Oryza sativa* plant cell causes it to be able to sequester in vacuole As bound to PCs **(Song *et al*., 2014)**. Negative effects of As have also been observed in agricultural production. Grain production of *Oryza sativa* and shoot biomass is heavily influenced by As toxicity **(Meselhy *et al*., 2021)**. As with other metals, As affect antioxidant enzymes such as CAT, SOD, APX and SOD as follows: clearly, when tested on two different varieties of *Oryza sativa*, it gave different results. In both varieties, an As concetration of 25 µM increased the activity of CAT, SOD, APX and POD. Differences only occurred at As concentration of 50 µM, where one of the varieties showed an increase in POD activity compared to the control, while the other showed a decrease. APX increased in both varieties, as SOD did. As far as CAT activities and an increase in the other but not a significant one **(Mousavi *et al*., 2020)**.

Nickel

Ni toxicity disrupts the equilibrium between the production of ROS and the antioxidant defense system, resulting in the accumulation of ROS and subsequent induction of oxidative stress. This heightened ROS production brings about changes in protein and lipid synthesis, enzymatic activities, leads to lipid peroxidation, DNA oxidation, and hampers cell division **(Hassan *et al*., 2019)**. The same effect has also Cd **(Hassan *et al*., 2020)**, Cr **(Wekeel *et al*., 2020)** and cooper **(Wang *et al*., 2023)**.

Copper

Maintaining Cu homeostasis is essential for the growth and development of plants **(Hu *et al*., 2023)**. In *Cassia angustifolia*, DNA damage was detected already at 10 mg/l of Cu **(Nanda and Agrawal, 2018)**. Hight amount of Cu significantly influences the level of proline and sucrose in plants and the activity of the antioxidant enzymes SOD, POD and CAT and hormones abscisid acid (ABA), indole acetic acid (IAA), salicylic acid (SA) and gibberellin acid (GA) in *Capsicum annum* **(Yuce *et al*., 2024)**. Additionally, the excess Cu triggered a rise in anthocyanin levels in *Malus domestica*. This was coupled with down-regulation in the expression of *miR408a* and Cu transport genes, while the expression of Cu-binding proteins like basic blue protein (BBP), laccase (LAC3) and Cu/Zn SOD superoxide dismutase (CSD1) was enhanced. This adjustment helped to regulate both Cu and ROS levels ensuring proper homeostasis **(Hu *et al*., 2023)**. Excessive amounts of Cu also affect the uptake of other nutritionally important elements such as potassium. In *Cucumis sativus*, potassium levels were reduced by up to 85% at a dose of 50 µM. Calcium levels were also reduced. An increase of phosphorus was observed, with values up to 298% higher than the control **(Feil *et al*., 2020)**.

Zinc

In the case of Zn, nicotianamine (NA) is very important for maintaining homeostasis in the plant **(Seregin and Kozhevnikova, 2023)**. Zn binds to nicotianamine, forming a complex. This complex is transported into the vacuoles by the zinc induced facilitator1 (ZIF1) transporter, thus eliminating Zn toxicity **(Yoneyama *et al*., 2015)**. In *Ipomoea batatas* (L.) Lam, application of 40 µM of Zn^{2+} can cause organelle death. Thus is an indication of severe stress and damage at the cellular level, which manifest as a reduction in root size and an alteration in root structure **(Meng *et al*., 2023)**. Zn causes changes in the parameters of photosynthesis and in the content of proline. However, these changes depend on the genotypes of the plant, as shown by studies on two different varieties of *Ipomoea batatas* (L.) Lam **(Meng *et al*., 2023)**. Genotype-dependent variations also have been noted in *Triticum aestivum* concerning their response to Zn stress **(Wei *et al*., 2022)**.

Mixtures of heavy metals

The accumulation of heavy metals in plants depends on the interaction between them. This interaction can be either synergistic or antagonistic. If several metals are present, the toxicity of these metals in plants is ultimately affected by this interaction. Here are some observations: In terms of accumulation, Cd and Cu seem to be in antagonistic relation. However, Cd affects Cu accumulation in the aerial parts of *Cucumis sativa*, reducing cooper toxicity. In contrast, Pb forms a synergistic relation with Cu in terms of accumulation in the root. Although Pb does not affect Cu content in the aerial part, it does impact the amount of Cd present in this part of plant **(An *et al*., 2004)**. Antagonistic relations have been observed between As and Zn and between Cu and As. Conversely, Zn and Cu show synergy when present together **(Gong *et al*., 2020)**.
Cd and Zn in *Nicotiana tabacum* plants led to alterations in Zn distribution within both shoot and roots. Specifically, at a Cd application of 4 µM and Zn concentration ranging from 5 to 50 µM, reduction in Zn distribution was observed. However, interestingly, this reduction varied inversely with the concentration of Cd and Zn; lower concentration of these metals corresponded to higher Zn distribution. This suggests a complex interaction between Cd and Zn and their impact on Zn distribution in *Nicotiana tabacum* **(Palusińska *et al*., 2020)**. Moreover, the presence of Cd and Zn induces changes in the uptake of other heavy metals in *Melissa officinalis*, notably manganese. Application of these heavy metals reduces manganese content and impacts the absorption of Cu and Pb. These findings prompt the authors to consider the possibility that Zn, and Cd compete with other the same transporters **(Adamczyk-Szabela *et al*., 2020)**. At concentrations of 8 µg/g Cd and 300 µg/g Zn, a significant improvement in various photosynthetic parameters (such NET photosynthesis, chlorophyll index and transpiration) was observed in *Melissa officinalis*. Interestingly, it was only at this specific combination of concentrations that this beneficial effect was observed. When each element was introduced separately, a decrease in these parameters was observed. This was even the case at lower concentrations of both elements when combined **(Adamczyk-Szabela *et al*., 2020)**.
The combination of Co and cooper affects the GSH and PC components in two different varieties in *Hordeum vulgare*. In the aerial part of the plant there is an increase of GSH under individual influences, but the combination of these two heavy metals increases GSH concentration even more. However, in the roots the opposite occurred and GSH decreased with the combinate effect. Although both varieties had the same behavior regarding GSH components, the difference was more visible in one variety than the other. The combination of two different heavy metals also affected the amount of PC in the root **(Lwalaba *et al*., 2020)**.

ACTUAL RESEARCH OF HEAVY METALS IMPACT USING MOLECULAR MARKERS AND TRANSCRIPTOMIC STRATEGIES

The function of mechanisms in plants after exposure to heavy metal has been extensively studied **(Küpper and Andresen, 2016)**. Modern research uses a variety of omic techniques. These include genomics, miRNAomics **(Jamla *et al*., 2021)** and transcriptomics to investigate the interaction between genes and heavy metals in plants **(Jamla *et al*., 2021)**. In addition to marker-based techniques such as inter simple sequence repeat (ISSR) **(Sherbeny, Morsi, Hassan, 2017, Sorentino *et al*., 2022)**, amplified fragment length polymorphism (AFLP) **(Sherbeny, Morsi, Hassan, 2017)**, random amplified polymorphic DNA (RAPD) **(Mengoni *et al*., 2000)**. There are also genome-wide association study (GWAS) analysis **(Pan et al, 2020, Derakhshani *et al*., 2020)**, RNA-seq analyses **(Derakhshani *et al*., 2020)** which can provide interesting results at the molecular level **(Angulo-Bejerano *et al*., 2021)**.

DNA marker systems in relation to heavy metal stress

DNA markers distinguish differences in nucleotide sequences between individuals or species. DNA markers are useful tools for the detection of individual genotypic differences – polymorphisms. These differences are caused by point mutations from single nucleotide substitutions, rearrangements involving insertions or deletions, DNA section duplication, translocations and inversions **(Amiteye, 2021)**. Arbitrarily amplified DNA markers are present at multiple sites throughout the genome and most of them are generated randomly over non transcribed and transcribed regions **(Poczai *et al*., 2013)**. These markers are usually used in phylogenetic studies, as a tool of marker assisted selection in plant breeding or for the construction of linkage maps **(Amiteye, 2021)**. Gene targeted and functional markers on the other hand target polymorphic sites within genes that affect phenotypic trait variations, these genes are usually present in multiple copies throughout the genome **(Poczai *et al*., 2013)**. Marker systems such CDDP (conserved DNA derived polymorphism) **(Collard and Mackill, 2009)** and PBA (cytochrome P450 based analogues) **(Yamanaka *et al*., 2003)** are stress sensitive methods, however according to the literature no studies are available today in relation to the influence of heavy metals on the genome of plants.

RAPD (Random Amplified Polymorphic DNA)

RAPD is PCR based methodology for analyzing DNA polymorphism, mode of inheritance is dominant, and it is not locus specific. The level of polymorphism which can be detected with this marker system is medium to high, however the reproducibility is low **(Amiteye, 2021)**. RAPD methodology is used in several studies to determine the genetic variation between plants exposed to heavy metals, and plants, which were not exposed to extra doses of heavy metals. The authors' results are not unambiguous, however, as biomarker of the present stressor (heavy metals) the methodology is able to capture genomic changes in plants. *S. alfredii* is a hyperaccumulator **(Wang *et al*., 2011)** of Cd native to China, RAPD investigation showed that in contaminated soils (Pb, Zn, Cd) near mines sites, the genetic variability is significantly lower than of normal populations grown in not contaminated soil, the reduction of genetic diversity can be caused by a strong bottleneck as a result of selection of heavy metal tolerant plants **(Deng *et al*., 2017)**. Studies of barley seedling *(Hordeum vulgare* L.) under Cd treatment showed changes in band intensity, as well as gain or loss of bands when compared to control **(Liu *et al*., 2009; Liu *et al*., 2005)**. Many more studies suggest that RAPD methodology is a good marker for heavy metal genotoxicity evaluation and that it is possible to clearly differentiate populations grown under heavy metal stress and populations without heavy metals stress. These studies include plants like mung bean- *Vigna radiata* L. (effect of Cu, Ni, Pb, Cd, Hg) **(Kumar *et al*., 2016)**, wild mint- *Mentha arvensis* L. (effect of Hg) **(Manikandan and Venkatachalam, 2011)**, roman nettle -*Urtica pilulifera* L. (effect of Cd) **(Dogan *et al*., 2015)**, *Silene paradoxa* L. (effect of Cu and Ni) **(Mengoni *et al*., 2000)**, common nettle- *Urtica dioica* L. (effect of Cu, Ni, Zn) **(Gjorgieva *et al*., 2013)**, *Arabidopsis thaliana* L. **(Conte *et al*., 1998)**, common bean- *Phaseolus vulgaris* L. (effect of Mn, Cu and Cd) **(Gjorgieva *et al*., 2012)**. Another study on common beans showed that a higher number of bands disappeared in RAPD profiles of plants treated with Cd and Cu than with Mn and Pb, when compared to untreated plants, though the difference between treated and untreated plants was evident in all samples **(Enan, 2006)**. DNA changes due to heavy metals are more detectable in plant roots than in leaves by RAPD methodology, as study on common beans showed **(Cencki *et al*., 2009)**. Accordingly leaves of *Tradecantia* plants after 0,5-1 year exposure to contaminated soil with heavy metals showed no polymorphic band patterns, only in two extremely hazardous soil sample grown plants **(Šiukšta *et al*., 2018)**. Results of the study on *Hibiscus rosa sinensis* showed that treatment with Cd and mixed metals manifested similar changes in band patterns, whereas plant samples treated with Zn and Pb were similar to control groups **(Bhaduri and Fulekar, 2015)**, studies mentioned above point to conclusion that from the studied metals Cd can cause the most changes in DNA priming sites according to RAPD methodology. There is also dose dependence, higher concentrations of heavy metals cause more changes in DNA priming sites, and lower doses resemble the band pattern of the not affected plants **(Aslam *et al*., 2014)**. Interesting result is the fact that in roman nettle plants, the detected DNA changes due to Cd contamination in parent plants, were transferred to the next generation **(Dogan *et al*., 2015)**.

SSR (Simple sequence repeats)

SSRs are simple sequences positioned in tandem repeat motifs, that are generously and randomly present in the genome of many species. They form a class of microsatellites and are present in the coding and non-coding parts of the DNA. The technique is PCR based, mode of inheritance is co-dominant, it is locus specific, the level of detectable polymorphism is high, and it is also highly repeatable **(Amiteye, 2021)**. SSR methodology is widely used in taxonomy and genome mapping studies, however in evaluation of genome changes and genotoxicity is less explored **(Monteiro *et al*., 2007)**. Only a few studies have been carried out to this day and the results are not unequivocal. SSRs may not be as widely useable markers of heavy metal toxicity as RAPD markers, another downside is the necessity of the study of SRRs present in the genome of the studied species. On the other hand, they are used in mapping studies to identify genes involved in heavy metal stress response **(Abou-Elwafa *et al*., 2019)**. SRRs were successfully applied in the study of Bermudagrass (*Cynodon dactylon* (L.) Pers.) populations from heavy metal pollution sites, mostly contaminated with Cd, Pb, Zn and uncontaminated sites. The genetic diversity of populations from contaminated areas was higher than that of metal free areas. The authors suggest that high number of tolerant individuals and gradual colonization events in natural populations can undo the initial genetic

bottleneck effect **(Xie *et al*., 2016)**. Populations of *Salix caprea* L., a heavy metal tolerant plant was examined from heavy metal contaminated and heavy metal free sites from Central Europe. A weak, but notable difference was observed between metallicolous and non-metallicolous populations. However, it should be mentioned that only 10% of the used SSR produced polymorphic profile **(Puschenreiter *et al*., 2010)**. SRR study of Cd induced stress in lettuce (*Lactuca sativa* L.) did not detect any changes in the genome of the plants tested, all the amplification products were monomorphic in leaf and root samples **(Monteiro *et al*., 2007)**. Authors **Mengoni *et al*. (2001)** recommended the technique of cpSSR (chloroplast SSR) as a useful complementary tool to RAPD for clarification of genetic differentiation patterns in heavy metal tolerant populations, as their results indicated genetic isolation of Cu tolerant populations of *Silene paradoxa* L. near Cu mines.

ISSR (Inter simple sequence repeats)

ISSRs is also a PCR based method, mode of inheritance is dominant, it is also locus specific, and the level of detectable polymorphism is high, however it has medium reproducibility. The methodology is more often used in genetic diversity studies than SSR, which is more a technique for genetic and association mapping **(Amiteye, 2021)**. The study of 3 medicinal plants **(El Sherbeny *et al*., 2017)** with ISSR primers was able to differentiate populations grown near cement factory sites, which are usually polluted with Pb, Cu, Cr, Cd, Zn **(Ogunkunle and Fatoba, 2014)** from non-polluted region-based populations. Comparable results were achieved by Słomka *et al*. (2011b), populations of *Viola tricolo*r L. growing on Zn, Pb and Cd contaminated soils showed higher gene diversity and polymorphism than control populations. ISSR analysis of rocket salad (*Eruca sativa* L.) with Zn, Pb and Cd treatment, detected a dose-dependent clustering and differentiation of heavy metal treatments from control groups **(Al-Qurainy, 2010)**. On the contrary, common duckweed (*Lemma minor* L.) a potential hyperaccumulator of Cd and Ni, showed high genomic template stability after heavy metal treatments, since low polymorphism was detected with ISSR and RAPD primers by authors **Ozyigit *et al.* (2021)**.

AFLP (amplified length polymorphism)

AFLP methodology can be applied to identify target genes for genotoxic agents in bioindicator plants and is used in risk assessments **(Labra *et al*., 2003)**. AFLP is a DNA fingerprinting methodology that can be applied to any organism. The technique is based on the digestion of genomic DNA with a mix of restriction enzymes followed by a selective PCR amplification of these fragments **(Vuylsteke *et al.*, 2007)**. Several studies have been conducted to study genetic diversity and identify genes associated with heavy metal stress with the help of AFLP methodology. Significantly different AFLP profiles were detected in *Arabidopsis thaliana* L. seedlings after Cd treatment **(Li *et al*., 2015)** in comparison to control plants, similarly in *Atriplex halimus* L., *Salsola tetramera* Botsch., E*chinops spinosissimus* subsp. *Spinosus spinosus* notable profile differences were observed between heavy metal polluted and non-polluted plants **(El Sherbeny *et al*., 2017)**.

In *Zea mays* L. AFLP band patterns showed substantial differences between unexposed and Cu exposed plants **(Qi *et al*., 2006)**. Contradicting results were recorded from AFLP analysis of 33 *Cistus ladanifer* L. populations from metalliferous and non-metalliferous areas, where no genetic differentiation between population types was observed **(Quintela-Sabarís *et al*., 2012)**.

CDNA-AFLP cDNA-AFLP analyses are also widely used to identify genes involved in heavy metal stress regulation. **Fusco *et al.* (2005)** detected 100 up-regulated or down-regulated gene fragments in *Brassica juncea* L. after Cd treatment. Results of **Zhou *et al*. (2013)** demonstrated that *Citrus sinensis* L. is more tolerant to Mn-toxicity than *Citrus grandis* L., and that Mn-toxicity affected gene expression more in roots than in *C. sinensis* leaves. The detected genes were homologous to genes with functions such as biological regulation and signal transduction, carbohydrate and energy metabolism, nucleic acid metabolism, protein metabolism and lipid metabolism, cell transport and cell wall metabolism. Another cDNA-AFLP study on Zn tolerance of *Sedum alfredii* L. also successfully identified genes with known or putative function **(Chao *et al.*, 2009)**.

Retrotransposon-based methodologies: IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism)

Transposon elements are mobile elements that are a large part of the plant genome. They function as gene expression regulators and can influence the evolution of the genome **(Hassan *et al*., 2024)**. The activation of transposons under abiotic or biotic stress is a well reported phenomenon. Transposons are classified based on the type of chromosomal movement and the nature of transposing units. We recognize conservative type movement (cut and paste) and replicative (copy and paste= retrotransposons). Retrotransposons are further divided into those with long terminal repeats (LTRs) and those without **(Negi *et al.*, 2016)**. LTR ends are profoundly conserved, what led to multiple marker systems that are based on retrotransposon sequences. However, only few studies are devoted to the detection of genome changes influenced by heavy metal stress, by these marker systems. IRAP methodology (inter- retrotransposon amplified polymorphism) is based on the distance between two retrotransposons with the use of outward facing primers that anneal to their LTRs sequences and REMAP methodology detects the sequence variation between LTR and a closely located SSR or microsatellite in the genomes of plants **(Amiteye, 2021)**. Studies showed similar patters as with other methodologies IRAP technique could distinguish heavy metal treated and untreated plants of *Zea mays* L. **(Taspinar *et al*., 2017; Yidiger *et al*., 2020)** and *Oryza sativa* L. **(Meriç *et al*., 2024)**, polymorphism showed as changing band intensity, gain or loss of bands, similar results were achieved by a study with REMAP methodology on *Zea mays* L. **(Yigider *et al.*, 2020)**. IRAP and REMAP techniques can also be classified as methodologies investigating epigenetic mechanisms as it was mentioned earlier that they intervene in the transcription of genes based on environmental factors **(Lisch and Bennetzen, 2011)**.

Table 1 Effect of heavy metals on plant genome assessed by genomic marker systems

Method	Heavy metal	Plant	Detected Effect	Citation
RAPD	Pb, Zn, Cd	*S. sedum alfredii*	loss of genetic variability	**Deng *et al*. (2017)**
RAPD	Cd	*Hordeum vulgare* L.	gain or loss of bands	**Liu *et al.* (2009)**
RAPD	Cu, Ni, Pb, Cd, Hg	*Vignata radiata* L.	variation in band intensity	**Kumar *et al*. (2016)**
RAPD	Hg	*Mentha arvensis* L.	gain or loss of bands	**Manikandan Venkatachalam (2011)**
RAPD	Cd	*Urtica pilulifera* L.	gain or loss of bands	**Dogan *et al*. (2015)**
RAPD	Cu, Ni	*Silene paradoxa* L.	high genetic variability	**Mengoni *et al*. (2000)**
RAPD	Cu, Ni, Zn	*Urtica dioica* L.	gain of bands	**Gjorgieva *et al*. (2013)**
RAPD	mixture of heavy metals	*Tradescantia* plants	low polymorphism	**Šiukšta *et al*. (2018)**
RAPD	Cd, Cu, Mn, Pb	*Phaseolus vulgaris* L.	variation in band intensity	**Enan (2006)**
RAPD	mixture of heavy metals	*Arabidopsis thaliana* L.	gain of bands	**Conte *et al*. (1998)**
RAPD	Cd, Pb, Zn	*Hibiscus rosa sinensis* L.	metal specific changes	**Bhaduri and Fulekar (2015)**
RAPD	Mn, Cu, Cd	*Phaseolus vulgaris* L.	gain or loss of bands	**Gjorgieva *et al*. (2012)**
RAPD	Cd	*Hordeum vulgare* L.	variation in band intensity	**Liu *et al*. (2005)**
RAPD	Hg, Cr	*Phaseolus vulgaris* L.	gain or loss of bands	**Cenkci *et al*. (2009)**
RAPD	Cd	*Capsicum annuum* L.	low polymorphism?	**Aslam *et al*. (2014)**
SSR	Cd, Pb, Zn	*Cynodon dactylon* (L.) Pers.	increased genetic diversity	**Xie *et al.* (2016)**
SSR	Cd	*Lactuca sativa* L.	monomorphic profiles	**Monteiro *et al*. (2007)**
SSR	Cd, Zn	*Salix caprea* L.	population differentiation	**Puschenreiter *et al*. (2010)**
cpSSR	Cu	*Silene paradoxa* L.	population differentiation	**Mengoni *et al*. (2001)**
ISSR	Pb, Cu, Cr, Cd, Zn	*Atriplex halimus* L., *Salsola tetramera* Botsch., *Echinops spinosissimus*	population differentiation	**El Sherbeny *et al.* (2017)**
ISSR	Zn, Pb, Cd	*Eruca sativa* L.	population differentiation	**Al-Qurainy (2010)**
ISSR	Cd, Ni	*Lemna minor* L.	gain of bands	**Ozyigit *et al*. (2021)**

ISSR	Zn, Pb, Cd	*Viola tricolor* L.	increased genetic diversity	**Słomka *et al*. (2011b)**
AFLP	Cu	*Zea mays* L.	Population differentiation	**Qi et al. (2006)**
AFLP	Cd	*Arabidopsis thaliana* L.	population differentiation	**Li *et al*. (2015)**
AFLP	mixture of heavy metals	*Atriplex halimus L., Salsola tetramera Botsch., Echinops spinosissimus*	population differentiation	**El Sherbeny *et al.* (2017)**
AFLP	mixture of heavy metals	*Cistus ladanifer* L.	no genetic variation	**Quintela-Sabarís *et al.* (2012)**
AFLP	mixture of heavy metals	*Viola reichenbachiana* L., *V. riviniana* L.	polymorphism	**Kuta *et al*. (2014)**
cDNA-AFLP	Cd	*Brassica juncea* L.	gene identification	**Fusco *et al.* (2005)**
cDNA-AFLP	Mn	*Citrus sinensis* L., *C. grandis* L.	gene identification	**Zhou *et al.* (2013)**
cDNA-AFLP	Zn	*Citrus sinensis* L., *C. grandis* L.	gene identification	**Chao *et al.* (2010)**
cDNA-AFLP	Co	*Sedum alfredii* L.	polymorphism	**Yigider *et al*. (2020)**
REMAP, IRAP	Ni	*Zea mays* L.	polymorphism	**Meriç *et al.* (2024)**
IRAP	Al	*Oryza sativa* L.	LTR RT mobilization	**Taspinar *et al.* (2017)**

Methods linked to epigenetic mechanisms

Adaptive – epigenetic processes represent events in the genome that, on the stimulus of the environment, can regulate the transcription of genes without rewriting the DNA sequences. These changes are stable and heritable. After stress exposure, an entire plant population can acquire a certain level of tolerance through epigenetic adaptive modifications **(Dutta *et al*., 2018)**. The main epigenetic mechanisms described are histone modifications on the chromatin level, on the genomic level DNA methylation and small RNA modifications **(Gallo-Franco *et al*., 2020)**. As for histone modifications, we know of histone acetylation, which increases access to DNA by neutralization of the basic charge in histones. The methylation of arginine/lysine in histones alters the accession for reading proteins **(Ueda and Seki, 2019)**. In plants, histone H3 is an important methylation target, specifically H3K9 lysine, associated with heterochromatin formation and H3K27 lysine, associated with developmental gene expression, also H3K4 and H3K36 methylation is linked to transcription promotion or repression, depending on the number of methyl groups that are added **(Fasani *et al*., 2023)**. Other mechanisms include phosphorylation **(Wang *et al*., 2015)** and monoubiquitination of histones **(Zhou *et al*., 2017)**. DNA methylation is a well-studied epigenetic mechanism, that is involved in a set of processes such as the activity of transposable elements, genomic imprinting, alternative splicing, and regulation of gene expression. In the *Oryza sativa* L. genome transposable elements and repetitive sequences are the most often methylated areas which give the genome stability. However gene expression regulation through gene promotor or gene body methylation is also frequent, in general plant DNA methylation is more complex and frequent than in mammals **(Gallo-Franco *et al*., 2020)**. Small non-coding RNAs are known epigenetic modulators which can affect protein levels without gene modifications. They work on the principle of base-pairing to their specific mRNA sequence, resulting in the initiation of silencing of mRNA translation **(Niekerk *et al*., 2021)**.

DNA methylation in response to heavy metal stress

DNA methylation is a well-documented epigenetic modification. DNA methylation maintains genome stability and gene expression and as a conserved epigenetic mark it can be inherited through transgenerational epigenetic inheritance. Some stress responses are memorized for a short period of time (hours, days) thought cell division, and in some cases the memory of stress exposure from parent plants are inherited to least the next stress-free generation though meiotic inheritance **(Liu and He, 2020)**. Gene stability is achieved by the fact that methylation acts as a defense mechanism against the jumping of transposable elements though the genome **(Zhang *et al*., 2011)**. Methylation is mediated during DNA replication and cell division though DNA methyltransferases **(Gallo-Franco *et al*., 2020)**. The process can be characterized by de novo methylation, 5meC recognition, and active and passive demethylation **(Czajka *et al*., 2021)**. As for the detection of DNA methylation, several research methodologies are used such as HPLC (high-pressure liquid chromatography), MSAP (methylation sensitive amplification polymorphism), AFLP (amplified fragment length polymorphism), CRED-RA (coupled restriction enzyme digestion-random amplification) and gene sequencing and methylation specific PCR (MSP) **(Erturk *et al*., 2015)** and others. It is also important to note that DNA methylation is dynamically regulated at the genomic level by demethylation processes, through DNA glycosylases. This regulation counteracts excessive methylation in different genomic regions and avoids the spreading of methylation in euchromatin **(Fasani *et al*., 2023)**.

CRED-RA (Coupled restriction enzyme digestion-random amplification)

CRED-RA is a technique for determining methylation status of the genome. The method has its foundation in the ability of methylation-sensitive enzymes such as Msp I and Hpa II to recognize the CCGG sequence, however MSp I is active only when methylation does not occur on the external cytosine and Hpa II when both cytosines are unmethylated **(Labra *et al*., 2004)**, after the digestion RAPD PCR follows **(Harshitha and Nair, 2020)**. The methodology is not so widely used for the detection of methylation status after heavy metal exposure, it is possible to find only a few studies that use this methodology in connection with heavy metals. The studies that were carried out, however, found the technique to be a useful tool for the evaluation of methylation status of plants under stress conditions, results showed that heavy metal stress causes hypermethylation in plants such as *Carthamus tinctorius* L. **(Bölükbaşı and Karakaş, 2023)** and *Zea mays* L. **(Erturk *et al*., 2014; Taspinar *et al*., 2017)**.

MSAP (Methylation Sensitive Amplified Polymorphism)

Methylation sensitive amplified polymorphism is a technique that has its basics in amplified length polymorphism (AFLP). It enables the analysis of whole genome methylation profiles. In plant genetics, it has been used to detect the level of genomic methylation, linked to chromatin structure regulation which controls gene expression **(Sun *et al*., 2021)**. The method also uses methylation-sensitive restriction endonucleases (MspI and HpaII) followed by amplification of the restriction fragments. The downside of the method is the difficult result interpretation, and the chance of inconsistency of the results with the current knowledge about methylation in plants **(Fulneček and Kovařík, 2014)**. The methodology was applied by several authors regarding stress associated with heavy metals. As for the result, several studies found elevated total methylation levels in plants after heavy metal treatment compared to control groups. As examples plants can be mentioned such as *Phaseolus vulgaris* L. **(Yildirim *et al*., 2023)** and *Arabidopsis thaliana* L. **(Wang *et al*., 2016)** after Cd treatment, and *Chenopodium ambrosioides* L. after Mn treatment **(Ding *et al*., 2023)**. Authors **Tang *et al*. (2022)** found significant correlation between total methylation levels and Cr treatment concentration in *Hibiscus cannabinus* L., furthermore, based on MSAP results 40 differentially methylated DNA sequenced fragments, turned out to have high homology to genes related to abiotic and biotic stress (ABC transporter F family member 3, RING-H2 finger protein ATL13-like, wall-associated receptor kinase-like 15, F-box protein At4g02760-like, basic helix-loop-helix protein A-like , 4-hydroxybenzoate polyprenyltransferase, tyrosine/DOPA decarboxylase 2-like). Similarly in soybeans (*Glycine max* L. Merr) a strong dose dependent correlation was found between Cd concentration and methylation polymorphism rates, while demethylation sites were similar to control groups **(Sun *et al*., 2021)**. There are also studies that have detected reduced overall methylation, such events were detected in *Trifolium repens* L. and *Cannabis sativa* L., which is a heavy metal tolerant plant, after Ni, Cd and Cr treatment **(Aina *et al*., 2004)**. **Ding *et al*. (2018)** reported changed full methylation and hemi-methylation profiles after Cd and Pb treatment in *Isoetes s Isoëtes inensis* Palmer. All authors confirmed epigenetic events that occurred as a response of the plant to heavy metals, MSAP can be a good monitoring methodology for these marks.

Table 2 Effect of heavy metals on the methylation status of plants assessed by different methods

Method	Heavy metal	Plant	Detected Effect	Citation
MSAP	Cd	*Phaseolus vulgaris* L.	elevated methylation level	**Yildirim *et al*. (2023)**
MSAP	Cd	*Arabidopsis thaliana* L.	hypermethylation	**Wang *et al*. (2016)**
MSAP	Al	*Triticale*	changes in methylation levels	**Bednarek *et al*. (2017)**
MSAP	Cr	*Hibiscus cannabinus* L.	increased methylation	**Tang *et al.* (2022)**
MSAP	Ni, Cd, Cr	*Trifolium repens* L., *Cannabis sativa* L. *Chenopodium ambrosioides* L.	hypomethylation	**Alina *et al*. (2004)**
MSAP	Mn	*Arabidopsis thaliana* L.	increased methylation	**Ding *et al*. (2023)**
MSAP	Cd	*Isoetes Isoëtes sinensis* Palmer	increased methylation	**Li *et al*. (2015)**
MSAP	Cd, Pb	*Glycine max* (L.) Merr.	changes in methylation	**Ding *et al*. (2018)**
MSAP	Cd	*Carthamus tinctorius* L	increased methylation	**Sun *et al*. (2021)**
CRED-RA	Cu	*Zea mays* L.	changes in methylation	**Bölükbaşı and Karakaş (2023)**
CRED-RA	Cr	*Zea mays* L.	dose dependent hypermethylation	**Erturk *et al.* (2014)**
CRED-RA	Al	*Zea mays* L.	hypermethylation	**Taspinar *et al.* (2017)**

Sequencing studies

The whole genome sequencing studies can help to map the methylation status of multiple genes at the same time, which provides us with information on the effect of heavy metals on the whole plant genome and its mechanism of self-defense. Multiple techniques are available today that can detect the methylation status of the plant genome. Genome-wide DNA methylation patters were investigated under Pb stress in *Zea mays* L. with methylated DNA immunoprecipitation-sequencing (MeDIP-seq), the analysis showed altered DNA methylation in 140 genes, among them were well-known stress-responsive transcription factors and proteins, such as MYB, AP2/ERF, bZIP, serine-threonine/tyrosine-proteins, pentatricopeptide repeat proteins, RING zinc finger proteins, F-box proteins, leucine-rich repeat proteins and tetratricopeptide repeat proteins **(Ding *et al*., 2014)**. Whole genome bisulfite sequencing (BS-seq) is a useful tool in methylation studies. Sodium-bisulfite converts unmethylated cytosines into uracil, while leaving the 5-methyl-cytosine residue intact **(Li and Tollefsbol, 2011; Frommer *et al.*, 1992)**. The method was used to detect methylation profile differences in TE in wild type (*Oryza glumaepatula*) and cultivated rice (*Oryza sativa* L.), the profiles between the species were mainly conserved with the presence of species-specific methylation patters, the study found several differentially methylated regions (DMRs) and DMR-associated genes that are linked to Al tolerance **(Gallo-Franco *et al*., 2022)**. **Cong *et al*. (2024)** with the help of BS-seq pointed out that Hg resistance associated genes in *Oryza sativa* L. Hg resistant lines are hypomethylated in their putative promoter regions.

miRNAs involved in heavy metal stress response

The uptake of heavy metals, their transport and detoxification including the associated physiological responses and phytohormone signaling are reported to be regulated by micro-RNA molecules **(Srivastava *et al*., 2012; Xu *et al*., 2019; Zhou *et al*., 2020)**. Micro RNAs (miRNAs) are small (18-24 nucleotides) non-coding RNAs that act as regulators and can cause mRNA destabilization and inhibition of translation **(Kumar, 2014)**. Micro RNAs can regulate the expression of other genes by two different pathways: transcriptionally (by methylation of target DNA, thus cause epigenetic modifications) and post-transcriptional (by cleavage or translation inhibition of target mRNA) **(Gielen *et al*., 2012)**. Plant miRNAs are involved in biotic stress responses (viral and bacterial) and abiotic stress responses (drought, high/low temperature, salinity, heavy metals) **(Kumar, 2014)**. The involvement of miRNAs in the responses of plants to heavy metals are functionally validated by different types of techniques **(Ding *et al.*, 2020)**. In literature, many miRNAs are reviewed to be responsive to heavy metal stress in plants (Table 3) and different processes were identified as involving in metal response through the miRNA's regulation, such as metal chelation, floral, root and leaf development or ROS detoxification **(Srivastava and Suprasanna, 2021)**.

Table 3 Examples of selected miRNA associated with heavy metal toxicity in plants

miRNA	Heavy metal	Plant	Reference
miR393	Al	*Hordeum vulgare* L.	**Bai *et al*. (2017)**
miR156, miR159, miR164, miR398, miR408	Cd	*Triticum aestivum* L.	**Qiu *et al*. (2016)**
miR390	Cd	*Oryza sativa*	**Ding *et al*. (2016)**
miR268	Cd	*Oryza sativa*	**Ding *et al*. (2017)**
miR166	Cd	*Oryza sativa*	**Ding *et al*. (2018)**
26 miRNAs	Cd	*Glycine max*	**Fang *et al*. (2013)**
miR167, miR393	Cd	*Zea mays*	**Gao *et al*. (2019)**
miR857	Cu	*Arabidopsis thaliana*	**Fu *et al*. (2019)**
miR444	Cr	*Oryza sativa*	**Sharma *et al*. (2015)**
miR54	As	*Brassica juncea*	**Qiao *et al*. (2018)**

Beside the studies of expression of miRNAs and identification of their target genes and functions **(Jones-Rhoades *et al.*, 2004; Yu *et al.*, 2012; Fu *et al*., 2019; Pandey *et al.*, 2020)**, the high conservation of miRNA sequences provided an opportunity to used them as an effective type of markers that is useful not only for genetic diversity study but also as potential biomarkers in plant stress responses **(Ražná *et al*., 2020)**. MicroRNA-based genotyping technique as a novel type of marker system was published in 2013 by authors **Fu *et al.* (2013)**. Two different types of plant miRNA-based DNA markers are recognized – first one are miRNAbased SSR markers reported in Fabaceae, *Oryza sativa* or *Punica granatum* **(Min *et al*., 2017; Patil *et al*., 2020; Tabkhkar *et al*., 2020)** and the second type are markers based on conserved regions of precursor miRNAs reported in *Gossypium* spp. or *Brassica* spp. **(Fu *et al*., 2013; Chen *et al*., 2013)**. Up to now, according to our knowledge, no specific studies have been published, where miRNAs-based markers were used to study the effect of heavy metals on their fingerprints, but their potential as stress specific markers can be assumed.

Expression profiles of gene families linked to heavy metal stress in plants

Using sequencing data, scientists can explore the whole transcriptome. RNA sequencing-based RNA-seq is a tool for transcriptome analysis **(Wang *et al*., 2013)**. This technology provides us immerse amount of information, which is subjected to bioinformatic analysis, facilitating the precise identification of differentially expressed genes (DEGs) **(Huang *et al*., 2024)**, sequence variation in identified genes, identify rare transcripts without prior knowledge of the gene, helping us to understand the complexity of regulation networks in plant stress management **(Wang *et al*., 2013)**. Microarray analyses are also popular, the technique can effectively monitor the expression levels of thousands of genes simultaneously **(Dalyan *et al*., 2017)**. To confirm gene expression, qPCR or northern blotting is used **(Kammenga *et al*., 2007)**. Multiple microarray studies are available that were carried out in connection with heavy metal stress, on *Brassica juncea* L. (Pb, Cd effect) **(Dalyan *et al*., 2017)**, *Arabidopsis helleri halleri* L. and *Arabidopsis thaliana* L. (Zn, Cd effect) **(Chiang *et al*., 2006)**, *Lycopersicon esculentum* Mill. (Cd, Cr, Hg, Pb effect) **(Hou *et al*., 2015)**. In effort to enhance plant tolerance to heavy metal stress, genetic engineering technologies rely on this information to strategically manipulate stress-responsive genes **(Ghosh and Roy, 2019)**.

To this day many studies have been carried out via qPCR or other earlier mentioned methods to monitor the effect of heavy metals on the expression levels of different genes. Examples of these studied and the effect detected by them are listed in Table 4. Heavy metals can alter expression of genes related to detoxification and antioxidant defense mechanism. Specifically, they can affect the expression of genes encoding detoxification enzymes or antioxidant enzymes. These include the

genes responsible for ascorbate-glutathione cycle, which includes important antioxidant enzymes such as MDAR, DHAR, GR and APX **(Adhikary *et al*., 2020)**. The influence of heavy metal on genes involved in sucrose synthase and tubulins was observed in *Oryza sativa* **(Ghouri *et al*., 2023)**. Chelators genes are also affected. But this change in regulation occurs depending on the type of heavy metal. In *Phoenix dactylifera*, Cr treatment induced greater changes in expression of the phytochelatin genes as compared to the metallothionein gene. Conversely, Cd exhibited a more visible impact on the expression of metallothionein gene compared to phytochelatin gene **(Chaâbene *et al*., 2018)**. As can impact the genes involved in anthocyanin biosynthesis pathway **(Jan *et al*., 2023)**. A family of plant Cd resistance (PCR) genes related to Cd resistance in plants have also been discovered **(Liu *et al*., 2023)**. By analyzing the distribution of cis-elements in bZIP gene in *Helianthus annus* L., it was found that those genes that ate rich in TC-repetitive elements in the promoter region respond to stress.

Table 4 Heavy metal effect on gene expression profiles in plants.

Gene family and method of detection	Heavy metal	Plant	Expression	Reference
Genes encoding antioxidant system				
BnGST (qPCR)	Hg	*Brassica napus* L.	Shoot, root ↑	**Yuan *et al*. (2021)**
BnPOD (qPCR)	Hg	*Brassica napus* L.	Shoot ↑, root ↓	**Yuan *et al.* (2021)**
BnAPX (qPCR)	Hg	*Brassica napus* L.	Shoot, root ↑	**Yuan *et al*. (2021)**
BsSOD (qPCR)	Hg	*Brassica napus* L.	Shoot, root ↓	**Yuan *et al*. (2021)**
APX-1 (qPCR)	Cr	*Zea mays* L.	Leaves (2-day, 50 mg/l, 4 day) ↑ (4 days, 100-200 mg/l) ↓	**Adhikari *et al*. (2020)**
GR (qPCR)	Cr	*Zea mays* L.	Leaves (4 day) ↑	**Adhikari *et al*. (2020)**
DHAR (qPCR)	Cr	*Zea mays* L.	Leaves ↓	**Adhikari *et al*. (2020)**
MDAR (qPCR)	Cr	*Zea mays* L.	Leaves ↑ (200 mg/l, 4 days) ↓	**Adhikari *et al*. (2020)**
Cu/Zn SOD (qPCR)	Cr	*Zea mays* L.	Leaves (4 days) ↓	**Adhikari *et al.* (2020)**
Genes encoding transporters of nutrients				
BnST (qPCR)	Hg	*Brassica napus* L.	Shoot, root ↑	**Yuan *et al*. (2021)**
BnPHO1 (qPCR)	Hg	*Brassica napus* L.	Shoot ↑, root ↓	**Yuan *et al*. (2021)**
ZIP genes family				
HabZIP40 (qPCR)	Cd	*Helianthus annus* L.	Leaves ↑	**Li *et al*. (2023)**
HabZIP96 (qPCR)	Cd	*Helianthus annus* L.	Root (0,3 g/kg Cd) ↑ (0,15 g/kg) ↓, leaves (0,15 g/kg) ↑	**Li *et al*. (2023)**
HabZIP71 (qPCR)	Cd	*Helianthus annus* L.	Root ↑	**Li *et al*. (2023)**
HabZIP16 (qPCR)	Cd	*Helianthus annus* L.	Root ↓	**Li *et al.* (2023)**
IbZIP1 (DEGs, qPCR)	Zn	*Ipomoea batatas* (L.) *Lam*	Root ↑	**Meng *et al.* (2023)**
IbZIP5 (DEGs, qPCR)	Zn	*Ipomoea batatas* (L.) *Lam*	Root ↑	**Meng *et al.* (2023)**
Ethylene-responsive transcription factor gene (ERF)				
IbERF110 (DEGs, qPCR)	Zn	*Ipomoea batatas* (L.) *Lam*	Root ↑	**Meng *et al*. (2023)**
Heavy metal associated (HMA) gene family				
HvHMA3 (qPCR)	Co	*Hordeum vulgare*	Root ↑	**Lwalaba *et al.* (2020)**
HvHMA5 (qPCR)	Cu, Cu+Co	*Hordeum vulgare*	Root ↑	**Lwalaba *et al.* (2020)**
Plant defensins (PDF) family genes				
BnaC2.PDF1.2a2 (qPCR)	Cd	*Brassica napus*	Root ↓	**Liu *et al*. (2021)**
BnaA7PDF1.2b2 (qPCR)	Cd	*Brassica napus*	Shoot ↑	**Liu *et al*. (2021)**
BnaC2.PDF2.2 (qPCR)	Cd	*Brassica napus*	Shoot ↑, Root ↓	**Liu *et al*. (2021)**
Genes of PCs				
Pdpcs (qPCR)	Cd, Cr	*Pheonix dactylifera*	Hypocotyl (time/concentration dependent)	**Chaâbene *et al*. (2018)**
Genes of MT				
Pdmt (qPCR)	Cd, Cr	*Pheonix dactylifera*	Hypocotyl (time/concentration dependent)	**Chaâbene *et al*. (2018)**
Anthocyanin biosynthesis pathway genes				
Pal (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al.* (2023)**
Chs (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al.* (2023)**
Chi (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al*. (2023)**
F_3H (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al*. (2023)**
Dfr (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al.* (2023)**
Ans (qPCR)	As	*Oryza sativa*	Root, Shoot ↑	**Jan *et al*. (2023)**

Genotoxic and mutagenic effects of heavy metals at the chromosomal level

Plants provide ideal genotoxicity assays for screening as well as monitoring of environmental mutagens or genotoxins, including heavy metals. Besides of negative impact of heavy metals on different levels of plant functioning particularly noticeable is their influence on cellular divisions, both mitosis and meiosis. Heavy metals induce structural chromosomal aberrations through breaks in DNA (clastogenic effect) or numerical chromosomal aberrations through interactions with cellular targets other than DNA, such as proteins involved in the segregation of chromosomes (aneugenic effect) **(Panda and Panda, 2002; Kumar and Srivastava, 2015)**. Mutagenesis affected by heavy metals in turn refers to gene or point mutation, a change in DNA sequence within a gene whereas recombinogenesis refers to homologous or non-homologous exchange of segments between chromatids or chromosomes. For instance, acentric chromosomal

fragments are the result of clastogenesis whereas lag chromosomes due to the aneugenesis **(Panda and Panda, 2002)**. Overall different kinds of abnormalities and disturbances are observed that include chromosome break or structural aberrations (clastogenesis), spindle malfunction affecting chromosome number (aneugenesis), micronuclei, sister chromatid exchange, and DNA strand break as evaluated by the Comet and Tunel **assays (Panda and Panda, 2002, Kwasniewska and Bara, 2022)**. The typical consequence is a reduced mitotic index and mixoploidy of the root cells both usually leading to reduced root growth **(Castro *et al.,* 2021)**. Some species, mostly crops (*Allium cepa*, *Vicia faba*, *Hordeum vulgare*, *Pisum sativum*, *Letuca sativa*, *Lycopersicon esculentum*, *Zea mays*, *Crepis capillaris*, etc.) serve as a well worked out assay systems with well-defined endpoints of genotoxicity. *Allium cepa* based, one of the most used, test is more efficient in detecting clastogenic changes while *Letuca sativa* rather detected aneugenic changes. Tradescantia micronucleus assay system has been validated and standardized for detecting disturbances in meiosis **(Fuchs *et al.,* 2018; Mišík *et al.,* 2019)**.

Different metals are used in these short-term exposure tests, both ballast ones (e.g. Pb, Cd, As, Cr) and micronutrients in increased thresholds **(Truta *et al.,* 2014)**. Usually, the lower the dose the lower genotoxic effect **(Aslam *et al.,* 2017)**. Plant assays are unique in the sense that they can be employed to evaluate genotoxicity of agents under wide range of environmental conditions that include *in situ* monitoring. Although metallophytes are of interest due to their increased threshold of tolerance to heavy metals, there are very few studies of the effects of metals on genome structure (karyotype). In facultative metallophyte *Viola tricolor* alerted karyotypes in root meristematic cells were observed which however did not corresponded with cDNA content in peduncles suggesting that these altered chromosomes were not involved into flower formation ensuring plant genetic stability **(Slomka *et al.,* 2011a)**. Similarly, particularly unstable karyotype seems to be attributed another metal tolerant plant – *Armeria maritima*, whose chromosome strong links at mitosis cause breaks generating chromosomal mutations and aneuploidy at the intra-individual level in all the populations **(Coulaud *et al.,* 1999)**. The role of metallo-adaptive response in evolution of metal tolerance in plants and its implication particularly in genecotoxicology of metals needs better understanding warranting further research.

ABBREVATIONS

ABA = abscisid acid
AFLP = amplified fragment length polymorphism
APX = ascorbate peroxidase
BBP = basic blue protein
BS-seq = bisulfite sequencing
CAT = catalase
CDDP = conserved DNA derived polymorphism
cpSSR = chloroplast SSR
CRED-RA = coupled restriction enzyme digestion-random amplification
CSD1 = Cu/Zn SOD superoxide dismutase
DEGs = differentially expressed genes
DHAR = dehydroascorabate reductase
DMA = dimethylarsinic acid
DMRs = differentially methylated regions
GA = gibberellin acid
GABA = y-aminobutyric acid
GR = glutathione reductase
GR = glutathione reductase
GSH = glutathione
GWAS = genome-wide association study
H_2O_2 = hydrogen peroxide
HMA = heavy metal transporting-ATPase
HPLC = high-pressure liquid chromatography
IRAP = inter-retrotransposon amplified polymorphism
ISSR = inter simple sequence repeat
ISSR = inter simple sequence repeats
JA = jasmonic acid
LAC3 = laccase
LTRs = long terminal repeats
MAPK = mitogen-activated protein kinases
MDA = malondialdehyde
MDAR = monodehydroascorbate reductase
MeDIP-Seq = methylated DNA immunoprecipitation-sequencing
MJ = methyl jasmonate
MSAP = methylation sensitive amplification polymorphism
MSP = methylation specific PCR
NA = nicotianamine
NADPH = nicotinamide adenine dinucleotide phosphate hydrogen
PBA = cytochrome P450 based analogues
PCs = phytochelatins
PDF = plant defensins
POD = peroxidase
RAPD = random amplified polymorphic DNA
REMAP = retrotransposon-microsatellite amplified polymorphism
ROS = reactive oxygen species
SOD = superoxide dismutases
SSR = simple sequence repeats
ZIF1 = zinc induced facilitator1

CONCLUSION

DNA markers are powerful tools in unraveling the complex genetic basis of plant responses to heavy metals. Their integration into advanced breeding, functional genomics, and synthetic biology holds transformative potential for both environmental remediation and sustainable agriculture. As genotyping technologies become more accessible, DNA markers will play an increasingly central role in developing plants tailored for contaminated environments or for safe food production and identification of Tolerance Genes and QTLs where helps identified candidate genes involved in uptake, transport, sequestration, and detoxification. An effective field for them is in use to assess natural variation in heavy metal tolerance among plant populations and identify tolerant genotypes in wild relatives or landraces for breeding programs. In the future, DNA markers will be used alongside other omics data to build regulatory networks for heavy metal response and will improve understanding of complex traits and enhances predictive breeding models.

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