

VARIOUS RESPONSES OF PLANT PROTEINS TO HEAVY METAL STRESS TOLERANCE

Meghna Chaudhary¹, Ritika Varshney¹, Mahima¹, Md. Shahnawaz¹ and Ram P. Pandey^{*1}

¹Department of Biotechnology, School of Basic and Applied Sciences, Sanskriti University, Mathura, India

*Corresponding Author Email- rprakash.pandey@gmail.com

ABSTRACT

Plants respond to various environmental hazards like heavy metal(s) by expressing genes that encode proteins involved in stress response. Toxic metal ions intensely affect the cellular protein homeostasis by interfering the folding and aggregation of native and non-native proteins leading to decreased cell viability. Though, plants contain a range of ubiquitous cellular surveillance systems that facilitate them to detoxify heavy metals toward enhanced tolerance to metal stress. As proteins comprise the key workhorses of living cells, the chelation of metal ions in cytosol with phytochelatins and metallothioneins followed by compartmentalization of metals in the vacuoles as well as the repair of stress-damaged proteins or elimination and degradation of proteins that fail to achieve their natural conformations are critical for plant to tolerate heavy metal stress. In this review, we present a wide overview of current advances in cellular protein research with regards to heavy metal tolerance in plants. We also discuss how plants uphold functional and healthy proteomes for survival under such unreliable environmental conditions.

Keywords: Heavy metals, Phytochelatins, Metallothioneins, Ubiquition proteasome system

INTRODUCTION

Proteins are categorized as macromolecules which play various roles in living organisms. They are involved in various cellular functions such as regulation, catalysis, cellular signaling and movement of nutrients and also provide structural support and protection to the cell (Amm et al., 2014). The structure of a protein determines its function which is obtained at the time of its synthesis. Additionally, the conformation of a protein depending upon the chemical and physical environment, which is largely affected by external environments such as reactive molecules, heavy metal (HM) ions and other stresses

(Goldberg, 2003; Amm et al., 2014; Zhou et al., 2016).

Rapid urbanization, industrialization and excessive application of insecticides and pesticides lead to increase in emission of pollutants into the environment. Since plants are sessile, they are widely face biotic and/or abiotic stresses including HM stress (Al-Whaibi, 2011).

Heavy metals bind to proteins, alter its native confirmation and thus, barricade its functionality (Hossain and Komatsu, 2013). Various heavy metals like methyl-mercury (MeHg) inhibits L-glutamine: D-fructose-6-phosphate aminotransferase, and overexpression of this enzyme confers

tolerance to MeHg (Naganuma et al., 2000). Correspondingly, thiol transferase activity is inhibited by cadmium (Cd) which binds to cysteine residues leads to oxidative damage. However, in *Brassica juncea*, Cd-dependent changes in beta carbonic anhydrase leads to increase in photorespiration which protects photosystem from oxidative damage (D'Alessandro et al., 2013). Cd disrupts the stabilizing interactions of tertiary structure of a protein which leads to loss of functions of that protein (Chrestensen et al., 2000).

Synthesis of peptides rich in metal binding properties take part in immobilizing and detoxification of metal ions (Clemens, 2006). This result into detoxification of HMs (Viehweger, 2014). However, in extreme conditions, cellular protein homeostasis is largely affected by metal ions which interfere with the folding process of proteins and accelerate aggregation of native or non-native proteins. This leads to endoplasmic reticulum (ER) stress and less cell viability. In order to prevent the aggregation of these proteins, cells possess various quality control systems. A special kind of protein known as heat shock proteins plays a key role in this system which mainly synthesized under stress conditions and maintain the cellular functionality (Amm et al., 2014). The damaged proteins which lost their native conformation and do not work properly undergo ubiquitin mediated degradation called as ER-associated degradation (ERAD). Another mechanism to eliminate these proteins occurs through autophagy (Liu and Howell, 2016). Although, plants contain a very strong mechanism to eliminate such unfolded proteins, still a very little information is available that how plants tolerate HM stress. In view of the above, we aim to explore a better understanding about the protein quality control system in plants with respect to tolerance of HM. We also try to uncover

the various mechanisms adopted by plants to ensure functional and healthy proteomes under HM stress.

Heavy Metals (HMs) Detoxification

Toxic metal ions generate reactive oxygen species at cellular level which creates oxidative stress to the cell (ROS; Li et al., 2016a). This result into DNA damage and/or failure in DNA repairs mechanisms. They also impede membrane functional integrity, nutrient homeostasis and also disturb protein function and activity (Tamás et al., 2014). Alternatively, plant cells are adopted to control excess metal ions and utilize various detoxification mechanisms to avert their participations in redundant toxic reactions. In the first line of defense, plants utilize strategies that prevent or reduce uptake by restricting metal ions to the apoplast through binding them to the cell wall or to cellular exudates, or by preventing long distance transport (Manara, 2012; Hasan et al., 2015). In contrast, when present at elevated concentrations, cells activate a complex network of storage and detoxification strategies, such as chelation of metal ions with phytochelatins and metallothioneins in the cytosol, trafficking, and sequestration into the vacuole by vacuolar transporters (Zhao and Chengcail, 2011).

Phytochelatins: Structure, Regulation and Function in Heavy-Metal Stress Tolerance

To avoid the damage caused by HMs, plants usually synthesize small cysteine-rich oligomers, called Phytochelatins (PCs) at the very beginning of metal stress (Ashraf et al., 2010; Pochodylo and Aristilde, 2017). Phytochelatins syntheses play a crucial role in tolerance to HMs in plants (Clemens, 2006; Emamverdian et al., 2015). It is observed that the biosynthesis of PCs can be regulated at post-translational level by metal(oid)s in many plant species. Though,

their over expression in plants not always gives rise to enhancement in tolerance to HM stress. For example, over expression of AtPCS1 in Arabidopsis, ironically exhibits hypersensitivity to the Cd and Zn; even though, PCs production is enhanced by 2.1-folds, as compared to wild type plants (Lee et al., 2003). As a matter of fact, excessive PCs levels in mutant plants accelerate accumulation of HMs like Cd without improving plant tolerance (Pomponi et al., 2006; Furini, 2012). This observable fact perhaps indicates few supplementary roles of PCs in plant cells, like their involvement in essential for sulfur metabolism, metal ion homeostasis and antioxidant mechanisms (Furini, 2012). Thus, prevention of toxic metals free movement inside the cytosol exhibits a latent mechanism for dealing with HM-induced toxicity (Hasan et al., 2016).

HMs detoxification mechanism is not only restricted to the chelation, but also do accumulate and stabilize HM in the vacuole of cells via formation of high molecular weight (HMW) complexes with PCs (Jabeen et al., 2009; Furini, 2012). Normally, metal ions sequestration is an adopted mechanism by organisms to ameliorate toxicity. The immobilized metal ions are transported to vacuole for sequestration through transporters. Vacuolar sequestration is very crucial machinery to HM homeostasis in plants, which is mediated by ATP-dependent vacuolar pumps (V-ATPase) as well as via tonoplast transporters (Sharma et al., 2016). Analysis of *de novo* transcriptome and RNA-Sequences exhibited that various genes that encode heavy metal ATPases (HMAS), ABC transporter and zinc iron permeases (ZIPs) are involved in the transport of metals as well as cellular detoxification (Xu et al., 2015; Sharma et al., 2016). Example of such kind of protein involved in uptake of Cd in *A. thaliana* is the Fe (II) transporter iron-regulated transporter 1 (IRT1) which belongs to the

ZIP family (Connolly et al., 2002). In addition, NRAMP5 which is the member of NRAMPs is documented as a crucial transporter for Mn acquisition and is also a major pathway for the entry of Cd into the roots of rice (Clemens and Ma, 2016). HM transporter 1 (HMT1) was first discovered in 1995 in the yeast *S. pombe*, as a vacuolar PC transporter required for Cd tolerance (Mendoza-Cózatl et al., 2011). Another transporter viz. HMA2 present in the plasma membrane of pericycle cells is responsible for transport of Cd to the symplast from the apoplast to facilitate translocation via the phloem in rice, while another transporter i.e. HMA3 in the tonoplast sequesters Cd into vacuoles by serving as primary pump (Clemens and Ma, 2016; Sharma et al., 2016).

Repairing of Damaged Proteins

HMs primarily targets the proteins. They either interact with functional side chain groups of proteins to form a complex with or relocate essential ions from metallo proteins which results into alteration in various physiological functions (Tamás et al., 2014). Additionally, HMs also alters the native confirmations of proteins by inhibiting folding process of native or non-native proteins that manifest a quantitative deficiency of the affected proteins which gives rise to the formation of proteotoxic aggregates (Bierkens, 2000; Tamás et al., 2014). Interestingly, in response to the stress, plants trigger the activation of different genes involved in cell survival and/or death in contaminated environmental conditions (Hossain et al., 2013). In response to such environmental conditions, a set of genes, known as stress genes, are induced to synthesize a group of proteins called HSPs (Gupta et al., 2010). In stress conditions, the induced synthesis of HSPs plays a major role to maintain the cellular homeostasis by accurate folding of nascent

and stress accumulated misfolded proteins, inhibit protein aggregation or by accelerating selective degradation of misfolded or denatured proteins (Hüttner et al., 2012; Park and Seo, 2015).

Role of HSPs in Plant Tolerance to HM Stress

Cellular stress created by HM often cause interruption to the cellular homeostasis because they inactivate essential enzymes and also suppress the functioning of proteins (Hossain et al., 2012b). Therefore, the induction of HSPs proteins is thought-out as a critical protective, ecophysiological adaptive and genetically conserved response of organisms in response to the environmental anxiety. Thus, they achieved crucial function in the antagonism of stress by re-establishing conformation of normal protein structure and cellular homeostasis (Rhee et al., 2009). Among the various types of HSPs, HSP70 family members have broadly been studied. Functional studies of HSP70 revealed that HSP70 is accumulated in response to environmental stressors in a broad range of plant species (Gupta et al., 2010). The specific members of this family are localized into the cytosol, mitochondria and endoplasmic reticulum(ER) and are constitutively expressed as well as regulated to maintain cellular homeostasis. For example, a member of HSP family, the 70-KDa heat shock cognates(HSC70) are constitutively synthesized in cells and frequently assist the folding of newly synthesized polypeptides and also take part in translocations of precursor proteins (Wang et al., 2004).

The current advancements in proteomics research have explained about the functional genes or proteins which involved in the responses of plants to HM stress at molecular levels (Ahsan et al., 2009).

Analysis of different transcripts in many plant species concluded that HSP70 is highly expressed under a variety of metal stress. Even though, various researchers concluded that the overexpression of HSP70 genes is positively correlated with the acquisition of tolerance to various stresses, including HMs. But the cellular mechanisms of HSP70 function under stress situation are not completely understood (Wang et al., 2004). HSP70 chaperones, together with their co-chaperones like DnaJ, make a set of prominent cellular strategies to check accumulation of *de novo* synthesized polypeptides as aggregates and ensure the proper folding of protein during their transfer at their appropriate locations (Al-Whaibi, 2011; Park and Seo, 2015). In transportation of precursor protein, the HSC70 is essential for cell-to-cell transport through interaction with the plasmodesmatal translocation pathway (Aoki et al., 2002). The induction of HSP70 not only limits the proteotoxic symptoms of metal ions, but also helps the sequestration and detoxification of these ions through MTs (Haap et al., 2016). Whereas the entire mechanism of HSPs-induced metal detoxification via MT has yet to be explored, only few studies pointed out that HSP60 might participate in protein folding and aggregation of various other proteins that are transported to organelles like mitochondria and chloroplasts (Al-Whaibi, 2011). Through our rising understanding of the proteome, it is clear that HSP60 is very crucial for cellular functions both at normal or stress environments, including metal stress. Interestingly, proteomics studies also revealed that the induction HSP60 chaperones prevents the denaturation of proteins even in the presence of metal ions in the cytoplasm (Sarry et al., 2006; Rodríguez-Celma et al., 2010).

Heavy Metals-Induced ER Stress

The currently synthesized and preexisting polypeptides present in the cell are always at a risk of misfolding and aggregation. It is observed that almost one-third of currently synthesized proteins are misfolded (Schubert et al., 2000). Additionally, cells constantly countenance various environmental challenges such as mutations, active oxygen radicals, heat and HM ions, which disrupt protein folding and also cause the misfolding of proteins which are already folded (Amm et al., 2014). The disturbance in proper functioning of the ER is mainly pertinent under stress conditions, while the requirement for secreted proteins exceeds its working capability and disturbs the normal functioning of ER, termed as ER stress (Schröder and Kaufman, 2005). Various researchers have documented that HMs and metalloids inhibit refolding of chemically denatured proteins *in vitro*, create hindrance in folding of proteins *in vivo* and also promote the aggregation of nascent proteins inside the cells (Sharma et al., 2011; Jacobson et al., 2012). It has been reported that in yeast, Cr can trigger oxidative protein damage and protein aggregation by enhancing mistranslation of mRNA (Sumner et al., 2005; Holland et al., 2007). Similarly, Cd has also been shown to cause the widespread aggregation of nascent protein and ER stress in yeast (Gardarin et al., 2010), whilst the exact mechanistic information of misfolding of proteins in the ER and cytoplasm remain to be uncover (Tamás et al., 2014). However, it could be related to metal-induced structural alteration of ER. Recently, Karmous and co-workers (2015) concluded that treatment of Cu in embryonic cells of *Phaseolus vulgaris* leads to prevalently swollen cisternae of smooth ER and vesicles with electrondense contents. Even though, this incident is often not clearly recognizable, it strongly checks the cellular homeostasis. Whereas, the toxicity of various metals, such as Cr, As, Pb, and

Cd is incontestable and interference of these HMs with the protein folding in living cells is clearly documented, the effectiveness of amassing of misfolded and aggregated proteins appears to be different (Tamás et al., 2014). In the cells of yeast, the accumulations of aggregated proteins occur in the order As>Cd>Cr upon the treatment of equal concentrations of these metals (Jacobson et al., 2012). The *in vivo* potency of these environmental hazards to prompt protein aggregation perhaps depends on the competence of their cellular uptake/transport and their different modes of biological action (Tamás et al., 2014). Under these conditions, a synchronized adaptive program known as unfolded protein response (UPR) is commonly initiated.

The UPR is a homeostatic response to tolerate ER stress via transcriptional and translational mechanisms. The stimulation of UPR is required for; primarily to restore the normal functioning of cell by inhibiting the production of secreted and membrane proteins, elimination of incorrectly folded proteins via ER-associated degradation (ERAD) mechanism and activation of the signaling pathways which are responsible to increased production of molecular chaperones involved in protein folding. If these cell sparing activities are not achieved within a certain time span or the disturbance is prolonged, the UPR aims toward programmed cell death (PCD) which is called apoptosis (Deng et al., 2013; Liu and Howell, 2016).

Degradation of Metal – Induced Denatured Proteins

The maintenance of cellular homeostasis, involves selective degradation of unnecessary misfolded or damaged protein which is essential for cellular functions, growth, development and viability. Degradation of protein or proteolysis can occur through various mechanisms such as

UPS (ubiquitin proteasome system) or autophagosome induction (Liu and Howell, 2016). UPS is a regulated mechanism which involve in the regulation of cellular protein homeostasis through various enzymatic cascade. Damaged protein degradation occurs rapidly , but sometime it occurs only in response to various cellular signalings (Pines and Lindon, 2005). The current studies concluded that UPS components controls the different processes in plants (Sadanandom et al., 2012). In Arabidopsis more than 6% protein coding genes are devoted to the UPS (Vierstra, 2009). In all eukaryotes , targeted proteins are marked for degradation by the attachment of ubiquitin. Such ubiquitylated protein recognized and then degraded into small peptides by the large proteolytic complex , the 26S proteasome (Goldberg, 2003). Ubiquitination is a highly regulated process which required energy in the form of ATP . It requires the action of three enzymes namely E1 (Ub -activating enzyme) , E2 (Ub – conjugating enzyme) and E3 (Ub- ligase) which work sequentially in a cascade (Maupin – Furlow , 2013). The first step in ubiquitylation , Ub covalently linked with E1 in an energy dependant reaction, the ubiquitin is first transferred from E1 to E2 through transesterification . Finally transfer of ubiquitin to the target protein from E2 is mediated by a enzyme E3 . This step is repeated to form polyubiquitinated chains and then designated for degradation which is mediated by 26S proteasome (Ruschak et al., 2011; sadanandom et al., 2012). Finally, polyubiquitinated chain is removed by deubiquitinating enzyme before import and proteolysis of proteins (Hartmann- Petersen et al., 2003).

UPS-Dependent Proteasome Activity and Metals Stress

Protein quality affected by increasing free radicals due to harsh environmental

conditions such as HM stress which create misfolding , denaturing and damage to protein. During stress , plant require a defense machinery to repair the damaged proteins or removed if damage is irreparable. In such situations, UPS plays a key role in plant response and adaptation to improve environmental conditions (Stone, 2014). The UPS function in nucleus as well as in cytoplasm which is responsible for removing most of the abnormal peptides and balanced the level of regulatory proteins and remove the temporary cellular regulators which may aggregate successive exposure to abiotic stress (Lyzenga and stone, 2012). The significance of UPS in cell has been recognized several years ago and manifested due to increased expression of polyubiquitin genes (Genschik et al., 1992; Jungmann et al., 1993). Expression of polyubiquitin genes under stressed environment indicates that UPS is involved in tolerance of heavy metal stress in plants (Sun and Callis , 1997; Chai and Zhang, 1998).Genetic analysis of rice plant exhibited that treatment with low concentrations of Cd induces polyubiquitin expression in root and shoot (Oono et al., 2016). Whereas, increased metal concentration causes the disturbance in proteasomal activity, which results in the deposition of abnormal proteins in the cytoplasm , which changes the cellular protein homeostasis and thus activate apoptosis (Yu et al. 2011). For example analysis of proteome in different species shows that ubiquitin activity reduced by Hg , Cu ,Cd ,Ni ,Co ,Pb and Cr at 100 μ M but not by Zn and Al , in contrast low concentrations can accelerate 26S proteasome activity (Aina et al., 2007; Pena et al., 2007, 2008). Even though, Cr , Pb , Cu ,Co ,Hg and Ni stimulate the aggregation of ubiquitin conjugated proteins , whereas abundance of 20S core protein in UPS is not altered (Pena et al., 2008).

Autophagy in Metal Stress Responses

In response to a wide range of harsh environmental stresses such as heat stress, drought, salt, nutrient starvation, oxidative stress and pathogen invasion plants have involved a defense mechanism called autophagy (Han et al., 2011; Wang et al., 2015; Xu et al., 2016; Yang et al., 2016). Though, its fundamental roles in plants, especially in HMs stress and adaptive responses, have perhaps not received the attention they deserve and thus leftovers elusive (Chiarelli and Roccheri, 2012; Pérez-Martín et al., 2015). Interestingly, in recent years, researchers begun to explore, the role of autophagy in plant for the tolerance to metals and the mechanism of adaptation is the same as in human and yeast. Recently, various researchers have documented that autophagy is induced in metal treated plants (Zhang and Chen (2010), Zheng et al. (2012)). It is observed that treatment of any the five different HMs (Cu, Ni, Zn, Cd and Mn) upregulates the 18 ATGs genes out of 30 by more than two folds in tobacco seedlings (Zheng et al. (2012)). They also explored that among the 18 ATGs, 11 ATGs are commonly upregulated in seedlings by all five metals, and the expression is more sensitive to Zn treatment than others. Recently, Abd-Alla and co-workers (2016) for the first time concluded that Ag-NPs treatments lead to the stimulation of autophagy in root nodules of *Rhizobium leguminosarum* as a mechanism of detoxification and surveillance. Altogether, current studies explored the involvement of autophagy as a sophisticated regulator of surveillance under HMs stress, nevertheless, the mechanisms, especially how metals regulate autophagy, still remain to be elucidated in the future (Zhou et al., 2015).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This review outlines the collision of HM stress on cellular protein homeostasis and illustrates the various mechanistic approaches that operate in the cells to regulate quality control systems in the direction of functional and healthy proteomes. Proteins are key workhorse of cells and are involved in the stress response in plants. HMs can activate various cellular pathways that are largely classified as death and survival signals. As surveillance mechanism, the ubiquitous plants response to HM stress is the chelation of toxic ions in the cytosol by cysteine rich peptides such as PCs and MTs, compartmentalization of metals in the vacuole by tonoplast located transporters, and the procedure that involves repair of stress-damaged proteins. The review of current research works exposed that MTs are not only required to complete the plants life cycle, but also play important roles in ionic homeostasis and allocation in plants as well as cleanup of ROS and confiscation of metals as that of PCs. At the same time as in extreme conditions metals intensely have an effect on cellular protein homeostasis by interfering with the folding procedure, they also arouse aggregation of native or non-native proteins leads to ER stress and decreased cell viability. Though, there is an archetypal set of proteins, called stress proteins or HSPs proteins, which are mainly expressed during stress. HSPs check the aggregation of nascent or non-native proteins and also activate repair of misfolded proteins. On the contrary, the damaged proteins which fail to achieve their native conformations are eliminated from ER by the activating ERAD machinery of ERQC system, leads to proteosomal (UPS) or autophagic degradation of such proteins.

Recent researches in protein exhibited that as core degradation process of misfolded or damaged polypeptides, the

over-expression of E3 enzyme in UPS pathway and the autophagic induction with mono-ubiquitination prevent metal accumulation in plants. However, it is still unclear the complete understanding of the mechanism of subsequent signaling cascades that control metal accumulation. The development of strategies to decrease metal uptake and translocation by manipulating cellular protein quality control system in plants seems to be promising approaches that potentially make sure the augmented yield as well as food safety.

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