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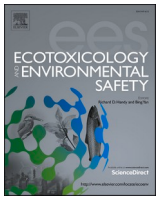

Review article

Biotechnological strategies for enhancing heavy metal tolerance in neglected and underutilized legume crops: A comprehensive review

Krishna Kumar Rai [a], Neha Pandey [a,b], Ram Prasad Meena [a,c], Shashi Pandey Rai [a,*]

[a] Centre of Advance Study in Botany, Department of Botany, Institute of Science, Banaras Hindu University (BHU), Varanasi 221005, Uttar Pradesh, India
[b] Department of Botany, CMP PG College, University of Allahabad, Prayagraj, India
[c] Department of Computer Science, IIT, Banaras Hindu University (BHU), Varanasi 221005, Uttar Pradesh, India

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ABSTRACT

Contamination of agricultural land and water by heavy metals due to rapid industrialization and urbanization including various natural processes have become one of the major constraints to crop growth and productivity. Several studies have reported that to counteract heavy metal stress, plants should be able to maneuver various physiological, biochemical and molecular processes to improve their growth and development under heavy metal stress. With the advent of modern biotechnological tools and techniques it is now possible to tailor legume and other plants overexpressing stress-induced genes, transcription factors, proteins, and metabolites that are directly involved in heavy metal stress tolerance. This review provides an in-depth overview of various biotechnological approaches and/or strategies that can be used for enhancing detoxification of the heavy metals by stimulating phytoremediation processes. Synthetic biology tools involved in the engineering of legume and other crop plants against heavy metal stress tolerance are also discussed herewith some pioneering examples where synthetic biology tools that have been used to modify plants for specific traits. Also, CRISPR based genetic engineering of plants, including their role in modulating the expression of several genes/ transcription factors in the improvement of abiotic stress tolerance and phytoremediation ability using knockdown and knockout strategies has also been critically discussed.

1. Introduction

Plants are sessile in nature therefore exposed to various challenging environmental conditions throughout their lifecycle that adversely affect their growth and developmental processes. Heavy metal toxicity reduces plant growth/productivity and causes severe health hazards in humans because of their accumulation in different parts of crop plants. Various metals and metalloids such as arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), nickel (Ni), zinc (Zn), cobalt (Co), aluminum (Al) and chromium (Cr) induce severe toxicity when they enter into the soil agro-ecosystem either through natural processes or by anthropogenic activities (Neilson and Rajakaruna, 2015). Preposterous anthropogenic activities have resulted in the increased pool of heavy metals that have augmented the level of the metals present in the earth's crust, therefore it is essential to discriminate between the naturally occurring metals from those that have piled due to human activities (Mohammed et al., 2011; Kumar et al., 2019). Natural sources of heavy metals contamination include weathering of rocks, soil erosion, burning of forests and volcanic eruptions whereas anthropogenic sources involve extensive mining, metal smelting, application of chemical fertilizers, industrial/sewage discharge and coal combustion (Luo et al., 2015; Singh and Kumar, 2017). Rapid industrialization and technological advancements have altered the normal geochemical cycle of metals/elements which in turn have accelerated their increment in soil horizons (Tak et al., 2013). Increased bioaccumulation of heavy metals beyond the threshold level has shown negative impact on the natural food chain and microbial flora, therefore is now being perceived as an imminent threat to the ecosystem and environment (Dudka and Miller, 1999; Sharma et al., 2007; Ali et al., 2013; Singh and Kumar, 2017; Saha et al., 2017).

Plants also need certain elements in trace amount as growth regulators or cofactors in cellular/biochemical reactions as well as are also a part of various proteins/biomolecules (Soetan et al., 2010; Hasanuzzaman et al., 2013). At higher levels these heavy metals may induce a toxic effect in plants thereby influencing their cellular, biological and molecular functions. Consecutively, plants have evolved sophisticated

* Corresponding author.
E-mail addresses: pshashi@bhu.ac.in, shashi.bhubotany@gmail.com (S.P. Rai).

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mechanisms for metal uptake, storage and their detoxification to neutralize the toxic effect of heavy metals (Hossain et al., 2012). The intracellular defense mechanisms in plants involve various enzymatic or non-enzymatic antioxidants, synthesis of compatible solutes and production of low molecular weight thiols/phytochelatins that are able to ameliorate toxic effect of heavy metals (Fig. 1) (Schwartz et al., 2010; Sytar et al., 2013; Gupta et al., 2014).

Underutilized legumes have significantly contributed towards the dietary requirements of the rural areas particularly under adverse environmental conditions such as drought and famine situations (Al-Snafi, 2017). These crops are the life-savers for millions of people where assuring food and nutritional security is one of the major challenges, particularly in ancestral subsistence farming activities (Naeem et al., 2020). Some of the known underutilized legumes are *Psophocarpus tetragonolobu*, *Horsegram* pp, *Phaseolus lunatu*, *Vicia faba,* and*Lablab purpureus* etc. that serve as a potential source of protein/fibers as compared to the other crop legumes such as *Glycine max*, *Vigna unguiculata*, *Cicer arentinum*, *Phaseolus vulgaris*, *Pisum sativum*, and *Arachis hypogaea* (Popoola et al., 2019). These underutilized legumes possess sufficient amounts of proteins/essential aminoacids, polyunsaturated fatty acids (PUFAs),vitamins dietary fiber, essential minerals and are also conceived as a medicinal legume playing important role in the treatment of various chronic diseases (Maass et al., 2010; Al-Snafi, 2017; Rai et al., 2018; Popoola et al., 2019). Apart from these, they are also well known for their adaptation under a wide range of climatic conditions thus labeling themselves as "climate smart crops" (D'Souza and Devaraj, 2013; Ruthrof et al., 2018; Rai et al., 2020b).

However, to date limited information is available regarding the adaptive mechanisms utilized by these underutilized legumes against heavy metal exposures and how they are able to colonize them for reclaiming lands by restoring soil fertility (Maass et al., 2010). Therefore, the present review is an attempt to evaluate the recent discoveries and breakthroughs made so far in deciphering different mechanisms/strategies that can be used by underutilized legume plants to tolerate/hyperaccumulate heavy metals stress. In addition, the present review will highlight physiological, biochemical and molecular responses associated with heavy metal tolerance and advancement made in different system/synthetic biology tools that can be exploited for improving the growth and productivity of underutilized legume plants under metal prone soils.

2. Heavy metal toxicity in underutilized legumes: status and prospect

Heavy metals at low concentrations play significant role in stimulating plant's growth and developmental processes by acting as cofactors

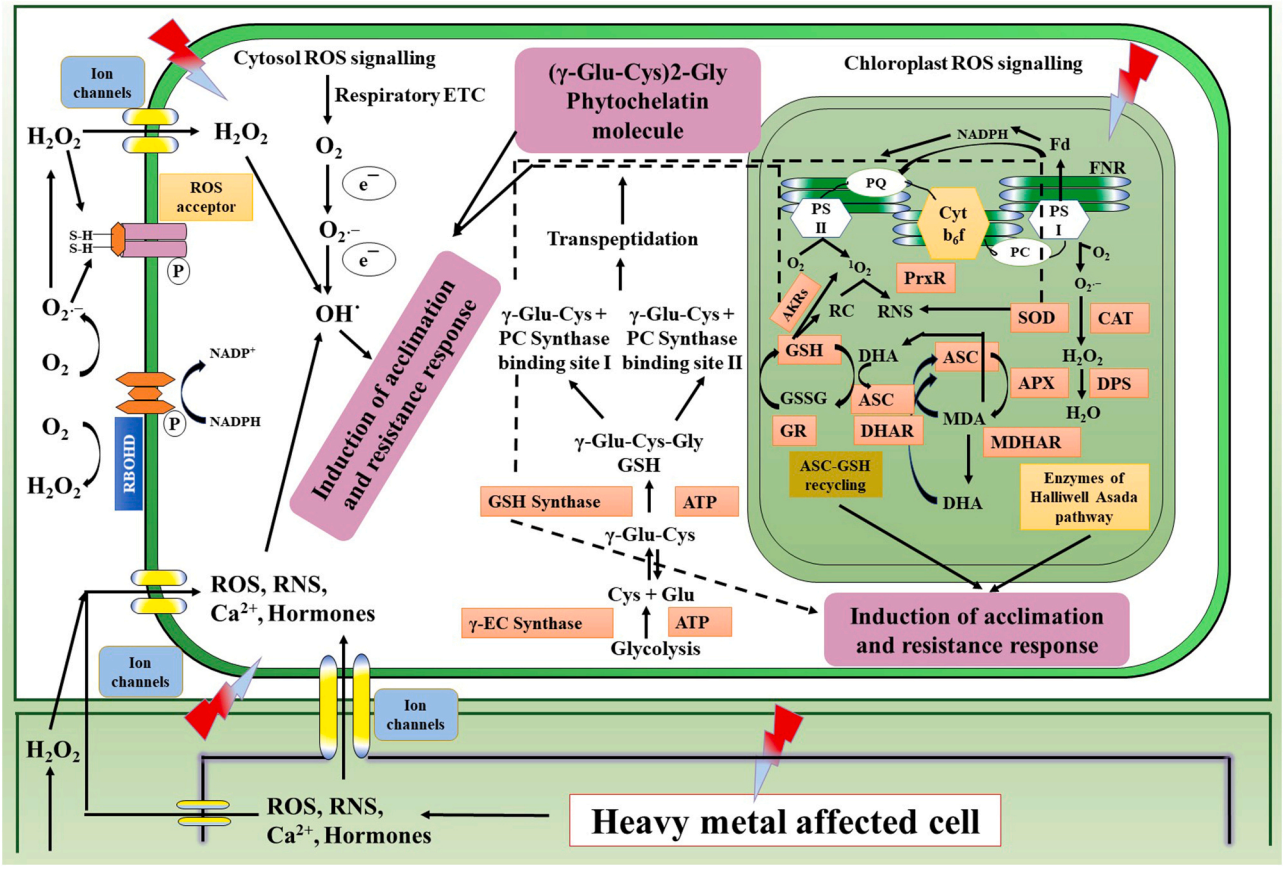

Fig. 1. ROS signaling in heavy metal stress affected plant cell. Abiotic stress imposes redox imbalance in different cellular compartments. ROS are continuously produced in cytosol and chloroplast as part of their normal cellular metabolism; however, abiotic stresses induce additional ROS generation including RNS and RCs thus causing redox imbalances which are sensed by ROS acceptor present in cytosol and migrate with in the cells through various ion channels. Despite of the complexity of ROS signaling, plants are able to sort out stress signals and activate their enzymatic and non-enzymatic antioxidant defense systems (both cytosolic and chloroplast antioxidative signaling simultaneously) in order to maintain optimal physiological conditions for their growth under heavy metal stress conditions. ROS: Reactive oxygen species, RNS: Reactive nitrogen species, RCs: Reactive carbonyls, GSH: Reduced glutathione, Glu: Glutamine, Cys: Cysteine, Gly: Glyoxalase, PC: Phytochelatins, AKRs: Aldo-keto reductase, PrxR: Peroxidoredoxins, DHA: Dehydroascorbate, GSSG: Oxidized glutathione, GR: Glutathione reductase, ASC: Reduced ascorbate, DHAR, Dehydroascorbate reductase, MDHAR: Monodehydroascorbate reductase, SOD: Superoxide dismutase, CAT: Catalase, DPS: DNA binding protein, Fd: Ferredoxin. FNR: Ferredoxin-NADP$^+$ reductase, RBOHD: Respiratory burst oxidase homolog decarboxylase, $O_2^{\cdot-}$: Superoxide anion, OH: Hydroxyl radical, $^1O_2$: Singlet oxygen, H_2O_2: Hydrogen peroxide, e^- electron, P: Phosphorylase.

of distinct enzymes involved in various physiological and metabolic pathways (Mohammed et al., 2011; Luo et al., 2015). However, when their levels reach beyond a certain threshold they also act as an imminent threat to plant growth and development which has become a worldwide issue (Pirselova et al., 2011). The family Leguminosae being one of largest and diverse plant families constituting over 700 genera and 20,000 species are being widely used as the most suitable plant species for phytoremediation of heavy metal induce toxicity in soils (Pajuelo et al., 2011). The legumes particularly *Glycine max, Phaseolus vulgaris* and *Lablab purpureus* are most commonly used for phytoextraction/phyto-stabilization mainly due to their ability to colonize metal-enriched soils to restore their fertility thereby stimulating crop growth and productivity (Ahmad et al., 2012). *Lablab purpureus* L. which is commonly known hyacinth bean or Indian bean have also been reported to be tolerant to several heavy metals such as Cd, Hg, Pb, Zn, P and Cr (D'Souzaand Devaraj, 2013; Ruthrof et al., 2018). Apart from legumes, several aquatic (*Pistia stratiotes, Spirodela polyrrhiza, Myriophyllum aquaticum*, and *Mentha aquatic*) and aromatic plants (*Chrysopogon zizanioides, Cymbopogon flexuosus, Cymbopogon martini, Bacopa monnieri*and *Cymbopogon winterianus*) have been extensively studied for their phytoremediation ability in heavy metal contaminated sites and have shown promising role in the accumulation of heavy metals. However, discussing both aquatic and aromatic plants is beyond the scope of the present paper as it focuses on underutilized legumes therefore, the readers are requested to read some excellent reviews discussing various aspects of phytoremediation in aquatic and aromatic plants (Muthusaravanan et al., 2018; Pandey et al., 2019; Tripathi et al., 2020).

Furthermore, studies have indicated that these underutilized legumes are able to perform phytoremediation and simultaneously boosting the nitrogen economy by forming a symbiotic relationship with *Rhizobium* thereby improving soil fertility and crop productivity (Ahmad et al., 2012; Ismaila et al., 2014; Stambulska et al., 2018; Naeem et al., 2020). Among legumes, model legume "*Medicago*" have been rigorously analyzed for its phytoremediation ability mainly because of its well-established genetics due to the availability of its whole genome sequence (Palma et al., 2013). Further, several genes linked to enhanced legume-*Rhizobium* symbiosis has been identified and simultaneously are also used for genetic manipulation of several soil microorganisms (Zaefarian et al., 2011; Amer et al., 2013; Chen et al., 2015b). Several studies have demonstrated that using *Medicago sativa* with *Sinorhizobium* (syn. Ensifer) *meliloti* and *Sinorhizobium medica* have significantly improved nodulation efficiency in *Medicago* plants which resulted in increased metal bioaccumulation via root nodules thus promoting land restoration and phytostabilization (Guinazu et al., 2010; Soto et al., 2010; Palma et al., 2013). Similarly, other reports demonstrated that using*Viccia faba, Lupinus luteus, Lens culinaris, Sulla coronaria* and recently *Lablab purpureus* with consortia of *Bradyrhizobium* sp. 750, *Pseudomonas* sp. Az13 and *Ochrobactrum cytisi* Azn6.2 had significantly improved plant growth and productivity while accumulating more heavy metals compared to non-inoculated ones (Dary et al., 2010; Guo and Chi, 2014; Lebrazi and Fikri-Benbrahim, 2018; Alam et al., 2019). However, concerted efforts are still needed to promote legume-based phytoremediation by rewiring host-plant symbiotic relationship through biotechnological interventions which are capable of regulating symbiont relation with host plants to increase metal tolerance and phytoremediation ability in plants.

3. Mechanism of heavy metal toxicity in plants

Heavy metals contamination of agricultural soils has become a daunting challenge around the globe as they induce severe detrimental effects on plant's growth and productivity as well as on animals consuming them (Afonne and Ifediba, 2020). Heavy metals such as Cd, Hg, As, Cr, Ni, Zn and other metals are required by plants in low quantity.At higher levels they adversely impact various physiological, biochemical and molecular process in plants causing membrane damage, reduction in photosynthetic rate, disruption of ion homeostasis, nutrient uptake and ultimately affecting nucleic acid and protein synthesis leading to growth arrest and finally death (DalCorso, 2012; Sarwar et al., 2017). Since, all the underutilized legumes are still considered as an "orphan crops" for their genome revolution and only limited reports have demonstrated regarding their heavy metal stress tolerance (Aritua et al., 2015; Rai et al., 2018) it is therefore imperative to decipher potential mechanisms of heavy metal toxicity in these crops.

3.1. Physiology and biochemistry of heavy metal toxicity

The toxicity of heavy metals has been attributed to different processes that are triggered simultaneously posing serious metal induce toxic effects via generation of oxidative stress (Prasad and Strazalka, 2000). Heavy metals toxicity results in the chelation of Mg^{2+} ions leading to inhibition of chlorophyll (Chl) synthesis or its degradation thus slowing down photosynthetic process, altered water transport, transpiration, stomatal movement and nutrient assimilation (Singh and Agrawal, 2010). The possible mechanism by which Zn^{2+} inhibits photosynthesis is by causing uncontrolled substitution of Mg-Chl with Zn-Chl via axial ligand binding property causing charge separation in photosystem II (PSII) (Ovecka and Takac, 2014). Further, the substitution of Mg^{2+} leads to protein denaturation thereby inhibiting the synthesis of ribulose 1,5- bis phosphate carboxylase/oxygenase (RUBISCO) protein and ultimately causing degradation of whole photosystems (Viehweger, 2014). The loss of chlorophyll degradation is the primary reason for visible chlorosis, reduced growth, necrosis and increased reddening of leaves due to enhanced anthocyanin production (Viehweger, 2014). Additionally, Cd^{2+} also replaces Mg^{2+} as the central ion and like Zn-Chl, Cd also replaces Mg^{2+} by axial binding and rapidly dissipating all the excitation energy as heat owing to its highly unstable singlet state and shorter lifetime (Prasad and Strazalka, 2000). Several researchers have reported that incorporation of Zn and Cd in PSII has caused a prominent decrease in fluorescence variable (Fv) and fluorescence maxima (Fm) leading to the loss of whole photosynthetic machinery (Jiwan and Ajah, 2011; Galland Rajakaruna, 2013; Ma et al., 2015). The toxicity of Cu^{2+} and Hg^{2+} has been intensely investigated in photosynthetic organism showever, these investigations are reported at a lower concentration. Still there are some important questions that can be deciphered by proper understanding of Cu^{2+} and Hg^{2+}toxicity at the higher level (Manara, 2012; Deng et al., 2016). Furthermore, all these heavy metals are also absorbed by the roots and transported to shoots causing significant damage to root and shoot cells, their morphology, internal organs like mitochondria and peroxisomes thus reducing energy production and ultimately death (Garg and Singla, 2011).

Higher level of heavy metals generally leads to the induction of oxidative stress in plants by tormenting various cellular and biochemical processes due to a dramatic increase in the level of reactive oxygen species (ROS). The levels of ROS increase dramatically because of the rapid flow of electrons due to the increased oxidation of metabolic activities in rapidly oxidizing organelles like chloroplast, peroxisomes and mitochondria (Prasad and Strazalka, 2000; Rastgoo and Alemzadeh, 2011). Rapid deterioration of water oxidizing complex of PSII (D1 and D2 proteins) by heavy metals instigate replacement of Ca^{2+} and Mg^{2+} thereby inhibiting PSII leading to uncoupling of the electron transport system in the chloroplast (Solanki and Dhankhar, 2011). Several researchers have documented the destructive role of heavy metals like Zn, Cd, Hg, Cr on important metabolic enzymes by causing conformational changes in their structures and enhancing metal substitution reactions thereby drastically reducing their catalytic activity (Huang and Wang, 2010; Piotrowska et al., 2010; Hossain et al., 2012). One of the reverberations of heavy metal stress on peroxisome is the increased mobility of peroxisome due to increase activity of peroxisomal enzymes such as xanthine oxidase, glycolate oxidase and flavin oxidase thereby triggering beta oxidation thus sparking the generation of ROS (Huang and Wang, 2010).

Plant mitochondrial electron transport system is considered as one of the ubiquitous sources of oxidative stress-induced ROS generation as it contains several dehydrogenases complexes capable of reducing the same pool of ubiquinone (Solanki and Dhankhar, 2011). In mitochondria, the primary site of ROS generation is Complex I, III and ubiquinone which are highly susceptible to heavy metals that induce chronic effect on their common respiratory functions (Hossain et al., 2012). In addition to mitochondrial complexes, heavy metals also trigger cell wall associated peroxidases and membrane bound-NADPH oxidases which are the two apoplastic enzymes that induce the generation of hydrogen peroxide and superoxide anion (Sytar et al., 2013). Further studies have demonstrated that the heavy metals such as Ni, Cd and Cr that have stimulated the activity of these two apoplastic enzymes which have resulted in the increased generation of ROS in *Pisum sativum*, *Viccia faba* as well as in *Arabidopsis thaliana* (Sharma et al., 2012). In addition, heavy metals can also lead to the generation of highly reactive hydroxyl radical (OH) via Fenton reaction which can cause extensive damage to cell membranes and nucleic acids (Sharma et al., 2012; Sytar et al., 2013).

3.2. Molecular biology of heavy metal toxicity

Deciphering metal toxicity and their physiological, biochemical and molecular responses is an extremely complex and challenging task at the cellular level. Several researchers are working extremely hard to unravel the actual mechanisms by which heavy metals induce a toxic effect on plants (Dalvi and Bhalerao, 2013). With the advent of modern biotechnological tools and technologies, it has now become possible to delve further and deeper to accomplish full insight into the metal-plant interaction (Kumar et al., 2015b). Advanced "OMICS" technologies have provided a better and complete system-wide view of physiological, biochemical and molecular processes of stressed cells (Kumar et al., 2015a). In this section we will provide a unique perspective of metal induced toxicity and its reclamation by regulation of proteomics, metabolomics and epigenomics changes in plants.

3.2.1. Proteomics mediated regulation of heavy metal toxicity

Plant response to heavy metal stress entails sever repercussions on plant's protein synthesis and expression. These changes not only include changes at the level of gene expression but also involve inevitable qualitative and quantitative modification in protein structure and their functions (Singh et al., 2016). Heavy metals can affect post-transcriptional modification, protein-protein interactions, splicing and protein translocation (Hossain and Komatsu, 2013). In addition, it can cause inhibition of protein expression by causing protein glycosylation and affecting other post-translational modifications such as methylation, phosphorylation, ubiquitination, biotinylation and sumoylation (Hossain and Komatsu, 2013). Comparative transcriptomics and proteomics analysis of rice plants has revealed several differentially regulated proteins/genes under Cd and As stress playing important role in the amelioration of toxic effect of heavy metal stress (Lee et al., 2010; Chauhan et al., 2020). In onion callus, significant increase was observed in the level of glutathione-S-transferase protein and decrease level of glyoxalase proteins upon exposure of Cd (0.5–1.0 mM) stress (Nahar et al., 2016a). Simultaneously, Zhao et al. (2011) also studied proteome changes in the leaf of metal accumulator plant *Phytolacca americana* exposed to 0.4 mM Cd stress. Their result indicated 11 genes were downregulated in which majority of them belonged to photosynthetic pathway and glutathione metabolism.

Likewise, cadmium stressed wheat root proteome analysis by 2-dimensional electrophoresis (2DE) showed 46 different protein spots (Yannarelli et al., 2007). Interestingly, they observed that most of the upregulated proteins belong to the antioxidant defense system whereas others resulted in downregulation of photosynthetic and electron transport system processes (Li et al., 2011). From proteomics study it has become well understood that heavy metals differentially affect the expression of proteins that are involved in cell signaling, stress detoxification, growth development and protein biosynthesis (Cherrad et al., 2012). Additionally, heavy metals also trigger ROS mediated oxidation and denaturation of distinct proteins involved in central metabolic pathway thereby impairing coordinated regulation of important cellular networks (Nwaichi et al., 2014; Shahzad et al., 2018). Similarly, researchers have also identified 40 differentially regulated spots in Al stressed *Glycine max*, and later characterized them using matrix assisted laser desorption ionization time of flight mass spectrophotometry (MALDI-TOF-MS). They identified that 21 proteins were upregulated which belong to antioxidant defense system, 14 newly accumulated and 5 proteins were downregulated (Zheng et al., 2008). Later they characterized and annotated 14 newly induced proteins functionally associated with signal transduction, cysteine synthase biosynthesis and sulfur metabolism which were further validated with the western blot analysis (Torres et al., 2013). Also, *Vigna unguiculata* L. plants exposed to Mn stress showed an enhanced generation of ROS in leaf apoplast and symplast that ultimately resulted in the oxidation of essential antioxidative enzymes such as catalase/peroxidase that ultimately lead to decreased photosynthesis and carbon assimilation (Fuhrs et al., 2008).

All these proteomic studies had identified potential protein markers which can be exploited as descriptors for differentiating tolerant genotypes from susceptible ones by analyzing the quantitative changes in the regulation of specific physiological and biochemical processes (Hossain and Komatsu, 2013). However, the challenge that still remains is the application of proteomic studies in several underutilized plants whose genome are not fully sequenced per se., *Lablab purpureus* L. Therefore, complete proteomic reference map has become an utmost priority which will help reveal important biological and molecular functions in the plants belonging to same genus or species. Nonetheless considerable progress has been made in this field that have facilitated our understanding of plant response to heavy metals stress and will pave the way for identification of candidate genes for developing stress resistant/tolerant plants/cultivars.

3.2.2. Metabolomics mediated regulation heavy metal toxicity

Several studies have been published in the recent years regarding heavy metal-influenced metabolome in both model plants as well as in cultivated genotypes (Singh et al., 2016). Among all the heavy metals cadmium stress is extensively studied using metabolomics approach in plants due to its frequent occurrence in the farmland as a direct consequence of industrial agriculture practices (Villiers et al., 2011). Effects of cadmium on *Arabidopsis* plants was analyzed by gas chromatography mass spectrophotometry (GC-MS) and revealed upregulation of metabolites such as glycine betaine, proline, glycerol and trehalose along with some antioxidant metabolites such as glutathione and ascorbate to counteract Cd-induced oxidative damages (Sun et al., 2010). In another study, liquid chromatography mass spectrophotometry (LC-MS) as a metabolomic tool was employed to study the Cd toxicity in old *Arabidopsis* plants. Different peaks obtained in LC-MS chromatogram were further analyzed by using multivariate statistics that led to the identification of several phytochelatins that were being synthesized upon exposure of Cd-stress (Sadi et al., 2008).

Metabolomics has also been intensively used in *Solanum lycopersicum* to decipher heavy metal stress response using nuclear magnetic resonance (NMR) technique, where *S. lycopersicum* plants exposed to varied levels of Cd-stress (Shulaev et al., 2008). Enhanced accumulation of citrate was observed in both leaves and roots which is involved in strengthening photosynthetic activity in leaves and metals chelation activity in roots. However, upon long-term exposure a significant decrease in the carotenoid and proline level was observed along with the decrease in glucose and fructose level in leaves which may have been due to metal induce inhibition of invertase activity (Hediji et al., 2010). Similarly, in *O. sativa* plants where both NMR and GC-MS metabolomics have been used in conjunction with an advanced transcriptomic tool to decipher the chronic effect of chromium stress (Wang et al., 2015b).

In-depth analysis of chromium exposed *O. sativa* plants showed extensive accumulation of proline and its plant's precursor ornithine which is likely to protect *O. sativa* plants from Cr-induced oxidative damages. In addition, a significant increase in the linoleic acid was also observed which is known to increase the synthesis of unsaturated fatty acid in plants thereby strengthening cell wall/membranes thus protecting cell death due to lipid peroxidation (Dubey et al., 2010).

It is evident from several metabolomics studies that it is a powerful technique to study plant metal interaction by serving a potential route for identifying novel stress-responsive metabolites and detoxification systems. Furthermore, it can also be used for deciphering novel metabolic pathways which can be efficiently linked with the existing pathways to fill the necessary gap by providing valuable information that can be used to generate a metabolomic map for future applications in those plants whose genome are not completely sequenced. Another major concern is the selection and identification of metabolic network that has been altered during the course of investigation because a change in the single metabolite involve in a myriad of pathways can trigger the alteration of the central metabolic pathway. Therefore, the urgent prerequisite is to pinpoint the pathway using a knowledge discovery approach or by performing a metabolome survey that will help in the interpretation of data and in building metabolic networks that can facilitate the identification of novel stress-responsive metabolites. Nonetheless, metabolomic hold the tremendous possibility to reveal novel and unique information that can be efficiently used to develop superior cultivars with enhanced heavy metal stress tolerance and phytoremediation ability.

3.2.3. Epigenomics mediated regulation of heavy metal toxicity

DNA methylation and nucleosome modification are the most common epigenetic modifications that are exclusively involved in the regulation of gene expression under different abiotic stress conditions (Peng and Zhang, 2009). DNA methylation not only regulate gene expression but also induces transposon mediated gene silencing/imprinting and does it by transferring a methyl group from S-adenosyl methionine (SAM) to 5'position of cytosine to yield 5'-methylcytosine (Khan et al., 2015). In plants, DNA methylation occurs at three sites CG, CHG and CHH (where H is any nucleotide) mediated by DNA methyl transferase-1, chromo-methyltransferase-3 and domains rearranged methyl transferase-2 (Du et al., 2012) and many other proteins. Several studies have already indicated that plant acquires tolerance to different types of abiotic stresses through acclimation processes mainly governed by epigenetic modification (Chinnusamy and Zhu, 2009; Kumar and Singh, 2016). Exposure of plants to several heavy metals has been shown to modify global DNA methylation pattern at whole genome level (Boyko and Kovalchuk, 2011). Researchers have applied whole genome bisulfide sequencing (BS-Seq) and chromatin immunoprecipitation technique to unravel changes in DNA methylation and histone modification at whole genome level both in *Arabidopsis* and *O. sativa* (Luo et al., 2012). Heavy metal stress induces a significant amount of DNA methylation in the *O. sativa* genome and such widespread changes can only be induced upon hypermethylation of DNA transposable elements in the vicinity of the stress-responsive genes (Ou et al., 2012). Various studies have indicated that heavy metals affect genome-wide DNA methylation in plants by causing condensation and O-phosphohomoserine reaction of SAM to form cystathionine and finally to homocysteine that affect sits synthesis by inhibiting sulfate assimilation pathway (Peng and Zhang, 2009).

Additionally, the carbon metabolism pathway also plays an important role in heavy metal stress-induced epigenetic changes including DNA methylation and histone modification (Cicatelli et al., 2014). This is most certainly because one-carbon carrier 5-methyltetrahydrofolate (5-CH3-THF) provides methyl group for the biosynthesis of Met by the activity of methylenetetrahydrofolate reductase (MTHFR) which require NADPH as a cofactor thus inducing epigenetic modifications under stress conditions (Dhar et al., 2014). A group of researchers tested this hypothesis in *Z. mays* plants exposed to heavy metal stress that exhibited reduced lignin levels as a direct consequence of reduced synthesis of SAM (Pawlak-Sprada et al., 2011a, 2011b; Grativol et al., 2012). Various studies have demonstrated the involvement of histone modification in the regulation of gene expression under heavy metal stressed plants. *Arabidopsis* plants exposed to heavy metal stress induces differential expression of nitrate transporter gene that caused trimethylation of H3 histone on 27th position of lysine (H3K27me3) at the higher level where as H3K4me3 and H3K36me 3 showed an opposite response (Yao et al., 2013). In another study, modification of H3K4 in the promoter region of MYB transcription factor (TF) downregulated the expression of several stress-responsive genes and resulted in the root hair elongation in heavy metal stressed *Arabidopsis* plants (Roy, 2016). Apart from histone methylation histone acetylation also regulates differential gene expression in plants exposed to heavy metal stress. Chen et al. (2015a) created a knockdown of histone deacetylase which resulted in decrease histone acetylation thereby altering the expression of stress-responsive genes in *Arabidopsis* plants exposed to heavy metal stress. However, the exact mechanism behind the histone modification in the regulation of gene expression in most of the plants remains elusive (Roy, 2016).

Growing shred of evidence have already confirmed the regulatory roles of epigenetic modification in controlling responses to heavy metal stress by modulating plant nutrient homeostasis (Grativol et al., 2012). However, the role of DNA methylation and histone modification are still limited to model plants and few crop plants. Therefore, deep efforts are required to unravel the actual mechanisms by which epigenetic modification rewires plant tolerance to a diverse range of abiotic stresses as most of the studies have only demonstrated DNA methylation as the basis of gene expression regulation (Chen et al., 2015a; Roy, 2016).

4. Strategies for improving metal tolerance and sequestration in underutilized legumes

The regulation/maintenance of ion and osmotic homeostasis in plant cells is an indispensable physiological condition for sustainable growth of plant and agriculture. Plants thriving under heavy metal stress conditions must adapt/acclimatize to avoid the severe effects of metal induce toxicity at all the three levels i.e. physiological, biochemical and molecular. The strategies by which the plants are able to cope up with heavy metal stress may be genotype specific or to some extent may be regulated by environmental conditions. Researchers working in the field of plant science community are still lagging in underpinning the complex mechanisms by which plants are able to survive under heavy metal stress conditions and simultaneously are able to maintain their growth and development without exhibiting a symptom of toxicity. This section briefly summarizes some recent developments in synthetic biology tools, the role of microRNA (miRNA), plant growth promoting rhizobacteria (PGPR) as well as recent advancement made regarding the role of salicylic acid and nitric oxide in the regulation of various aspects of heavy metal stress tolerance in plants.

4.1. Stimulation of antioxidant defense machinery – salicylic acid and nitric oxide mediated regulation

Salicylic acid (SA) and nitric oxide (NO) are ubiquitous phytohormone and signaling molecule that play a broad-spectrum role in the regulation of antioxidant defense system in response to multiple abiotic stresses (Rai et al., 2020b). Both SA and NO modulate antioxidant defense system in plants by regulating the biosynthesis of ROS and their effective scavenging by inducing transcriptional changes and expression of stress-responsive genes involved in various signal transduction processes, cell growth and development (Gemes et al., 2011). A large number of reports have confirmed the regulatory role of both SA and NO in protecting plants from the abiotic stress-induced oxidative damages by maintaining an effective balance between ROS production and scavenging (Rai et al., 2018, 2020b, 2020a). Also, both SA and NO have

been reported to protect the cell membranes from lipid peroxidation by regulating the activity of lipoxygenase (LOX) enzymes and by stimulating the production of osmo-protectants such as glycine betaine and proline (Singh et al., 2017). In the past recent year, a heavy metal hyper accumulator plant *Solanum nigrum* has caught the eyes of researchers working in the field of plant metal response. The roots of the plants have been shown to accumulate NO in response to heavy metal stress thus protecting the plant from the heavy metal induce oxidative damage (Xu et al., 2010a). Several studies have reported that exogenous application of SA and NO are effective in improving plant tolerance to a wide range of abiotic stress (*S. lycopersicum,* L. *purpureus,Cicer arientinum, Zea mays, T. aestivum*, and *O. sativa*) including heavy metals (*Arabidopsis*, *Thlaspi* and *Helianthus annus*) by regulating the activities of several enzymatic and non-enzymatic antioxidants (Cabello et al., 2014; Kong et al., 2014; Tripathi et al., 2017).

SA and NO have been reported to accumulate various low molecular weight molecules such as polyamines which are ubiquitous to all living organisms able to influence a variety of growth and physiological processes in plants (Xu et al., 2011; Wang et al., 2013). Researchers have indicated that polyamines also act by interacting and stimulating glyoxalase system thereby enhancing the biosynthesis various non-enzymatic antioxidants such as ascorbate and glutathione. Thus, stimulating the scavenging of ROS along with methylglyoxal (MG) which is a cytotoxic dicarbonyl aldehyde compound whose level is dramatically increased under abiotic stresses especially in heavy metal stress (Li, 2016). In addition, MG also induces chromatin modification and endoreduplication by forming advance glycation end products (AGEs) stimulating chemical modifications and protein denaturation (Nahar et al., 2016a). The MG detoxification pathway consists of glyoxalase system involving two key enzymes of glyoxalase pathway: glyoxalase I and glyoxalase II (Ghosh et al., 2016). These ubiquitous enzymes are actively involved in the conversion of MG into 2-hydroxyacids using reduced glutathione (GSH) as cofactor which is then converted to S-D-lactoylglutathione (SLG) and finally to D-lactate via two step reaction recycling GSH back into the system (Ghosh et al., 2016). Recent studies have indicated that plants can instantly improvise at physiological, biochemical and molecular level environments by activating glyoxalase system against various abiotic stress conditions including heavy metals (Nahar et al., 2016b). Several studies have also indicated that SA and NO are also involved in the activation of various TFs such as WRKY, NAC (No apical meristem, *Arabidopsis* transcription activation factor and Cup-shaped cotyledon), DREB (Dehydration Responsive Element Binding) protein, AP2/ERF (Apetella 2 and Ethylene Responsive Factor). These TFs which play significant role in triggering the activation of various stress-responsive genes involved in plant defense against heavy metal stress as well as genes involve in cellular response, apoptosis, detoxification and nutrient assimilation (Erpen et al., 2018). In addition, SA and NO have been reported to stimulate the synthesis of heat shock protein (HSPs) known as molecular chaperones in response to various biotic and abiotic stress conditions as well as in response to heavy metal stress (Song et al., 2012). Several lines of studies have indicated that exogenous application of SA and NO as well as their intracellular synthesis have led the induction of HSPs in response to several heavy metals such as Cd, Cr, Ni, Al and Hg (Song et al., 2012; Rai et al., 2020b). In another study, researchers recorded a steep increase in the expression of HSPs in *Armeria maritima* and *Glycine max* plants exposed to Cd and Al stress (Xu et al., 2010b). The activation of these molecular chaperones is essential for maintaining proper structure and correct folding of proteins in response to stress conditions and simultaneously channeling them for their effective degradation by modification of their ubiquitin system (Lal, 2010).

4.2. Stimulation of heavy metal transporters and chelators proteins

The uptake and maintenance of heavy metal homeostasis in a cell is regulated by the activity of specific metal transporter proteins and metal pumps (Manara, 2012). These transporter proteins/metal pumps are membrane proteins belonging to different protein families which are regulated both at tissue (transporting metal ions from mesophyll)and organ (transporting metal ions from root to shoot) levels (Oveckaand Takac, 2014). At the subcellular level, these transport proteins regulate the transport of metal ions across the plasma membrane as well as among other organelles.The ZIP family of metal transporter proteins are exclusively involved in the uptake and accumulation of Fe and Zn in plants (Manara, 2012). However, the proteins of this family are also regulated in response to Cd, Cr and Ni stress response (Thapa et al., 2012). *Arabidopsis* has been reported to contain three ZIP transporter proteins expression of which are activated in response to elevated Zn levels thereby stimulating tolerance level by regulating ion homeostasis (Assunçao et al., 2010). The ZIP transporter protein identified in model legume *Medicago truncatula* (ZIP 6) and *Glycine max* (ZIP 1) has suggested their role in the controlled regulation of Zn and Fe (Lopez-Millan et al., 2004; Bamrungsetthapong et al., 2010).

Heavy metal associated transporters; a member of the P1B-ATPase family has also been reported to regulate metal ion transport from the plasma membrane to the cytoplasm as well as from root to shoot (Rastgoo et al., 2011). Recent studies have indicated that they contain metal chelating activity and their homologous/heterologous expression can stimulate heavy metal stress tolerance in plants as they have been predicted to have broad substrate specificity and are linked to Hg, Zn, Co and Cr tolerance (Rastgoo et al., 2011). Overexpression of *AtHMA4* has been shown to improve the growth and development of *Arabidopsis* plants by stimulating root growth under Zn, Cd and Co stress conditions (Barabasz et al., 2016). This hypothesis was also confirmed by creating a knockout of heavy metal ATPase (*AtHMA4)* that exhibited increase sensitivity towards heavy metal stress (Siemianowski et al., 2011). Similarly, in *Medicago truncatula*multidrug and toxic compound extrusion (MATE) genes *MATE* 1, 2, 55, 66 and aluminum-activated malate transporters 4 (*ALMT4)* gene in *Lotus japonicus* have been overexpressed to confer heavy metal stress tolerance (Wang et al., 2017b). Another member which belongs to metal transporter family of proteins is cation diffusion facilitators (CDFs) whose primary function is to act as antiporters of Zn, Cd, Ni which are located in vacuolar compartments (Gustin et al., 2011). Researchers have created transgenic *Arabidopsis* plant overexpressing microsomal triglyceride transfer protein (*AtMTP1*) gene that has shown remarkable tolerance to heavy metal stress as compared to wild type (Tanaka et al., 2015). Similarly, researchers have also created transgenic *Medicago truncatula* and *Glycine max*overexpressing molybdate transporter type 1.3 (*MOT1.3*) and Divalent metal transporter 1 (*DMT1*) genes that have shown enhanced tolerance against excess Fe and Mo levels (Escaray et al., 2019).

Another class of transporter protein is natural resistance-associated macrophage protein (NRAMP) that encodes many integral membrane proteins that mediate metal ion transport across plasma membranes (Pottier et al., 2015). A study has delineated that overexpression of *AtNRAMP1–4* and *OsNRAMP1*can perform effective transportation of Fe thus increasing the tolerance level of *Arabidopsis* and *O.sativa* plants (Mitra, 2015). Researchers have intercepted the location of *OsNRAMP1*to plasma membrane/pericycle cells and have created transgenic *Arabidopsis* plants overexpressing *OsNRAMP1* gene that have shown enhance tolerance to As and Cd by accumulating them in root and shoots. Similarly, in another study Meena et al. (2018) have confirmed the regulatory role of NRAMP proteins in the regulation of heavy metal stress tolerance in *S. lycopersicum* plants using *in-silico* approach. However, till date no NRAMP proteins have been identified in legume plants therefore concerted efforts are needed to discover these key proteins for the proper understanding of metal stress response in legume plants.

The low molecular weight cysteine-rich proteins metallothionein (MT) have been implicated in detoxification of heavy metal in plants (Freisinger, 2011). This family protein has been known to exert their function by their ability to scavenge ROS as their overexpression (*AtMT2a* and *AtMT3*) in *Viccia faba* and *Arabidopsis* have shown

enhanced tolerance to Cd stress by protecting lipid peroxidation of membranes (Xia et al., 2012). Similarly, overexpression of *BrMT1* from *Brassica rapa* has increased the tolerance of *Arabidopsis* plants exposed to Cd stress by effectively maintaining their photosynthetic apparatus (Ahn et al., 2012). Recently, Tsyganov et al. (2020) have shown that metallothionein protein (*PsMT1* and *PsMT2*) from pea (*Pisum sativum*) plants allowed to create an overexpressing strain of *Rhizobium leguminosarum* that successfully increased the tolerance and accumulation of Cd in wild pea plants by stimulating nodules formation. Apart from these, phytochelatins synthase (PCS) are also a group of metal-binding proteins that are accumulated in plants and in some marine alga under heavy metal stress conditions (Yadav, 2010; Navarrete et al., 2019). Phytochelatin functions by chelating heavy metals with their thiol group thus forming a metal-phytochelatin complex which is then effectively sequestered in vacuoles (Yadav, 2010). Several phytochelatins have been identified in various plants for example transgenic *O. sativa* plants overexpressing *T. aestivum* phytochelatin protein *TaPCS1* showed increased absorption of Cd in root and shoot thus enhancing their Cd-stress tolerance (Wang et al., 2012). Similarly, researchers have confirmed that various phytochelatins from *Arabidopsis*, *Medicago* and other crop plants if co-expressed with cysteine desulfydrase, can offer great biotechnological advantage for tailoring crop plants for heavy metal stress tolerance (Pal and Rai, 2010; Sunitha et al., 2013).

4.3. Application of PGPR in metal toxicity

Past recent years have witnessed the extensive application of plant growth-promoting rhizobacteria (PGPR) particularly those that can colonize roots. Therefore, researchers have diverted their attention towards the interaction between plants and soil microorganisms for enhancing heavy metal tolerance in plants (Kang et al., 2010). The synergistic relation between soil microorganism and plant roots have been widely reported to significantly induce the remediation processes using arbuscular mycorrhizae (AM) as well as other growth promoting rhizo and endo bacteria (Liu et al., 2012). The AM have been reported to form a symbiotic relationship with plant thus improving their nutrient assimilation, stimulating their growth and development in heavy metal contaminated soil by enhancing their accessibility to nutrients like P and K. Additionally, they also help in maintaining appropriate soil texture by preventing soil leaching and by accumulating heavy metals in their shoots, roots and leaves and converting them into non-toxic form (Sarathambal et al., 2017). Besides, AM fungi have been reported to increase metal translocation and sequestration in various legume plants as well as in sunflower plants thus efficiently promoting phyto-stabilization and revegetation of contaminated soils (Mishra et al., 2016). On the contrary, several endophytes and PGPR have been extensively employed to stimulate phytoremediation ability of various plants including both model legume and crop legumes by stimulating the biosynthesis of several organic acids, siderophores and plant growth regulators (Gururani et al., 2013; Mishra et al., 2016).

Additionally, PGPRs like *Arthrobacter, Microbacterium, Bacillus, Kocuria and Pseudomonas* spp. have known to play a significant role in solubilizing various metals for their enhanced sequestration in metalliferous soil where metals are very tightly bound to the soil by secreting protons and other organic anions such as oxalate, acetate, citrate and glycolate or by acting as biosurfactants (Singh and Singh, 2013). Further, PGPR can stimulate the biosynthesis of siderophores under the iron-deficient condition which acts as iron chelators thus promoting iron availability to both soil microorganism and plants. For instance, *Brassica juncea* plants supplemented with a mutant form of phosphate solubilizing *Enterobacter* sp. NBRI K28 showed increased production of siderophores which ultimately enhances growth and development of *Brassica* plants by stimulating root cell proliferation, increased production of root hairs and Indole-3-acetic acid (IAA) synthesis (Ahemad, 2015). Several researchers have tested and approved PGPRs as potential bio-inoculum for enhancing remediation of contaminated soil. For example, heavy metal mobilizing bacteria*Rahnella* sp. JN6 isolated from *Polygonum pubescens* have been shown to improve tolerance of *Brassica napus* plants exposed to heavy metal stress (He et al., 2013). Likewise, Tonin et al. (2001) have exploited AM fungi along with non-accumulator metallophyte *Viola calaminaria* to stimulate the heavy metal stress tolerance in maize plants grown under heavy metal contaminated soil by promoting plant–microbe interaction. In another study, researchers had identified and isolated rhizosphere bacterial consortium from pseudometallophyte *Betula celtiberica* residing in As contaminated soil that significantly promoted growth and development of various plants including legumes exposed to As stress by increasing their phytoaccumulation ability (Becerra-Castro et al., 2012).

PGPR and phytoremediation are emerging eco-friendly 'green-clean' technology that has tremendous potential for remediation of heavy metal contaminated soil to increase agricultural productivity and farmer's livelihood. However, detail biological and molecular mechanisms of plant–microbe interaction in most of the plants is still vague and need to be uncovered. Furthermore, most of the endosymbiotic bacteria residing in the complex rhizosphere are yet to be characterized and cultured for their effective implementation in bioremediation and phytoremediation program. With the advent of various biotechnological tools and techniques both system and synthetic biology can be extensively employed for deciphering molecular mechanisms behind plant–microbe interaction and the role they play in the cleaning of heavy metals and organic pollutants. However, the application of PGPR and AM fungi in the stimulation of phytoremediation is still scarce and limited to few plant species. Therefore, efforts are required to expand the implementation of both PGPR and AM consortia to those plant species whose genomes are not fully sequenced as a result of which the potential of phytoremediation remains unexploited in various underutilized legumes including hyacinth bean.

4.4. Application of synthetic biology tools in metal toxicity

Synthetic biology is a next-generation biotechnology discipline that intercepts advanced molecular biology from a rational perspective and tries to revamp the existing biological systems (Hagemann and Hess, 2018). In the past recent years, research on synthetic biology was primarily centralized on model organisms, such as *E. coli* and yeast which unraveled several key biological mechanisms to further the understanding of these model organisms for the production of useful products (Adams, 2016). Synthetic biology tools can also facilitate genetic modifications through zinc finger nucleases (ZFNs), transcription activators like effector nucleases (TALENs) and recently discovered clustered regulatory interspaced short palindromic repeats (CRISPR)-CRISPR associated protein (Cas). Despite these technological breakthroughs, the barrier does exist for its implementation inimproving plant-metal response (Mani and Kumar, 2014). However, one of the major bottlenecks in the engineering of plants for heavy metal stress tolerance using synthetic biology tools is the presence of a smaller number of phytobricks (parts used in synthetic biology) as compared to bio bricks-their microbial counterparts (Fig. 2, Table 2). This section mainly discusses the basic principle behind the application of synthetic biology in plants concerning heavy metal tolerance with some pioneering examples of the development of phytobricks and genome-scale models (GSMs).

4.4.1. Inducible and constitutive promoters

An ideal inducible promoter should lie dormant upon the absence of inducer, able to generate a foreseeable response, should be stable under different phases of growth and development of hosts, and most importantly should not affect host cell's transcription (Behle et al., 2019). Earlier studies have reported that the majority of inducible promoters are often been characterized for their metal-binding activity that helps host cells to limit their metal intake under different oxidative stresses (Grefen et al., 2010). For example, a promoter named coaA and PnrsB from *Synechocystis* sp. *PCC 6803* and Psmt and PisiAB from

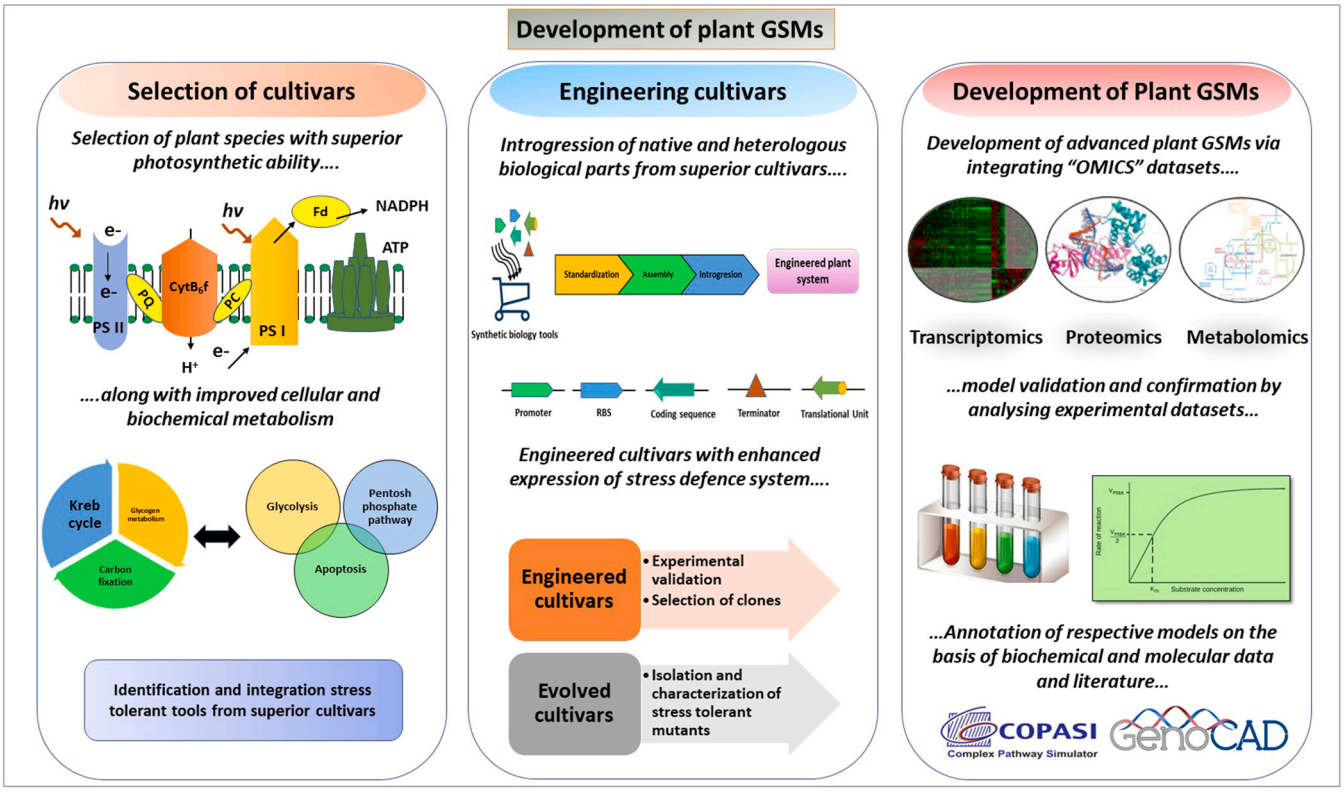

Fig. 2. Strategies for the development of Genome-Scale Models (GSMs) in plant system. Earlier developed models were solely dependent on physiological and biochemical datasets thus limiting their efficiency to carbon metabolism and photosynthetic reaction. Technological advancements have scaled-up the generation of advanced and improved model. Continuous upgradation of several publicly available databases (GenoCAD, Copasi, KEGG and SynBioSS) have substantially contributed towards the development of synthetic biology tools. However, still certain refinements can further improve the quantity and quality GSMs like identification and introduction of new stress tolerant genetic elements from model plant system to cultivated plants and once the genetic elements and its effects are bioinformatically and experimentally validated, it could results in the development of more sophisticated GSM that could predict cellular, metabolic and molecular responses with high accuracy and efficiency.

Table 1
Some recent sRNA predictive tools identified in plants exposed to heavy metal stress.

Plant species	Type of sRNA tool	Target gene	Sequencing tool used	Alignment tool used	Gene regulation	Functional role	References
Oryza sativa	miRNA (OsmiR601, 602, 604, 605)	Cytochrome P450 family protein, Ribosome proteins	Sanger sequencing	Not mentioned	Positive regulation	Regulation of growth and development in response to Cd stress	Huang et al. (2009)
Arabidopsis thaliana	miR397, miR398a, miR408, miR857	Growth regulatory factors	RNA sequencing	Bioconductor Package, PlantDARIO	Positive regulation	Regulation of antioxidant defense machinery under Cu stress	Banerjee et al. (2016)
Arabidopsis thaliana	*miR158, miR159, miR164, miR172,*	Transcription factors	Differential RNA sequencing	Bioconductor Package, PlantDARIO	Positive regulation	Regulation of antioxidant defense machinery under Fe stress	Banerjee et al. (2016)
Sorghum bicolor	*miR166, miR171, miR172,*	Osmotic stress responsive genes	RNA sequencing	Bioconductor Package, PlantDARIO	Positive regulation	Regulates photosynthesis process under Zn stress	Banerjee et al. (2016)
Paeonia ostii	miRNA	Lipid transfer protein	RNA sequencing	SOAP2	Positive regulation	Regulation of antioxidant defense machinery under Cu stress	Jin et al. (2015)
Medicago truncatula	miRNA156/157, miR319	MAPK cascade genes	RNA sequencing	Not mentioned	Positive regulation	Regulation of growth and development in response to Hg and Cd stress	Zhou et al. (2012)
Phaseolus vulgaris	miR319	Phytohormone signaling gene	RNA sequencing	Not mentioned	Positive regulation	Regulation of antioxidant defense machinery under Mn stress	Valdes-Lopez et al. (2010)
Arabidopsis thaliana	siRNA	WRKY genes	RNA sequencing	FASTX-Toolkit	Positive regulation	Regulation of growth and development in response to Cu and Cd stress	Ma et al. (2018)
Raphanus sativus	miRNA	MAPK cascade genes	High-Throughput sequencing	Rfam	Positive regulation	Regulation of plant adaptive responses to Pb stress	Wang et al. (2015a)

Table 2
Comprehensive list of developed genome-scale models (GSMs) in plants.

Plant species	Genome based model	Target genes/metabolites	Functional role	Algorithm used	References
Arabidopsis thaliana	AraGEM,	Primary metabolic network	Improved growth and enhanced synthesis of biochemicals	Flux balance analysis	De Oliveira Dal'Molin et al. (2009)
Zea mays	iRS1563	Metabolic pathways	Improved pyruvate metabolism and cellular growth	Flux balance analysis	Saha et al. (2011)
Oryza sativa	CK1721	Genes involved in light response	Regulation of photosynthesis	^{13}C metabolic flux analysis	Poolman et al. (2013)
Zea mays	C4GEM	C4 carbon fixation and investigate nitrogen (N) assimilation	Regulation of photosynthesis and photorespiration	Flux balance analysis	Simons et al. (2014)
Oryza sativa	CK1721	Inorganic macronutrients	Improved growth and enhanced synthesis of secondary metabolites	Flux balance analysis	Chatterjee and Kundu (2015)
Oryza sativa	iOS2164	Glutathione-ascorbate metabolism	Increased activity of the malate valve under drought stress	Flux balance analysis	Lakshmanan et al. (2013)
Arabidopsis thaliana	AraCore	Carbon metabolism	Reduction in carbon-use efficiency at the higher temperature	^{13}C metabolic flux analysis	Williams et al. (2010)

Synechococcus sp. *PCC 7942* was induced (up to 300–1000-fold) upon their exposure to Co^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+}. Several other inducible promoters have been widely used in plant systems to study heavy metal toxicity (Singh et al., 2016). Interestingly, transgenic *Brassica juncea* lines over-expressing glyoxalase I gene under the control of rd29A promoter and cauliflower mosaic virus promoter (CaMV35S) showed enhanced tolerance to salinity (200 mM NaCl), heavy metal (5 mM Zn) and drought stress (200 mM mannitol) compared to non-transformed plants (Wu et al., 2013). Similarly, *Arabidopsis* multidrug resistance-associated protein 3 (*AtMRP3*) promoter and *AtPCS* has been shown to regulate the expression of several stress-responsive genes and transcription factors in response to Cd, Ni, As and Co (Bovet et al., 2005; Tsai et al., 2012). Recently, several researchers have attempted to improve the growth and biomass of tomato plants by increasing the heavy metal detoxification upon overexpressing *LeHSP23.8* gene which is a strong heat-inducible promoter (Tao et al., 2015). So, in the future, these promoters can be exploited for heterogenous production of heavy metal detoxifying enzymes in other plant species for improving their growth and biomass accumulation. Studies have indicated that most of the inducible promoters are designed by stimulating the interaction between response regulators (RRs) and TFs to modify the function of several target genes (Nguyen et al., 2016). Engineering of RRs encoding genes and TFs encoding genes such as DREB, AP2/ERF, WRKY, NAC can tremendously enhance the tolerance against heavy metal stress (Liu et al., 2014). Now the efforts are needed to identify and develop other native inducible promoters with the ability to regulate plant growth and defense response under heavy metal stress.

Constitutive promoters have been widely demonstrated in plants for enhancing their growth and productivity under various abiotic stress conditions by efficiently modulating the expression of multiple genes of stress defense pathways (Gao et al., 2014). Various promoters viz., *PjMT1* and *PjMT2* have been systematically elucidated for their ability to target multiple genes to induce heavy metal stress tolerance in transgenic *N. tabacum* plants (Chaudhary et al., 2016). Comprehensive efforts are being made to develop strong promoters either by modifying the existing promoters or by developing new promoters from available online RNA-deep sequencing databases. A modified tandem promoter *AtPCS1* from *Arabidopsis* when fused with *CaMV35S* promoter resulted in increased tolerance of transgenic *Arabidopsis* plants by maintaining a sufficient level of GSH pool compared to their wild counterpart (Parvin, 2018). Similarly, several endogenous promoters have been modified/optimized such as *CaMV35S*and *rd29A*for efficient regulation of functional antioxidative enzymes as well as the expression of other stress-responsive genes up to 10-fold (Rajwanshi et al., 2016). Several independent studies have confirmed that the truncated promoters could work more efficiently and effectively compared to their wild form (Rajwanshi et al., 2016). Recently, researchers have successfully designed strong promoter *CaMV35S* with the help of sophisticated bioinformatic tools like bTSS finder and virtual footprint in *Arabidopsis* plants.The promoter was able to regulate the expression of *O. sativa* metallothioneins *OsMT3a*up to 8-fold compared to original ones that showed enhanced tolerance to salinity (NaCl), drought (PEG) and metal ($CdCl_2$) stress (Mekawy et al., 2018).

4.4.2. Riboswitches as function for gene regulation

Riboswitches have presented themselves as a potential tool for efficiently controlling gene expression by maneuvering secondary structures of target mRNA. All naturally occurring riboswitches are aptamers that inflict conformational change in the secondary structure of mRNA and thus impacting their translation efficiency (Bocobza and Aharoni, 2014). The aptamer sequences of riboswitches function both as *cis* and *trans*-acting sequences that either have activating or repressing effect on the target mRNA. Natural riboswitches exist as stem-loop structures that prevent their efficient binding to 5' ribosome binding site (RBS). On the contrary, binding of certain trans-acting metabolite or RNA to aptamer riboswitches bring significant change in its conformational state that stimulates their accessibility for the ribosome and eventually translation of target protein (Verhounig et al., 2010). In prokaryotic system riboswitches have bestowed themselves as a powerful functional genetic tool for efficient regulation of gene expression with a high degree of modularity. In addition, they are also capable of driving the expression of several proteins over large distances even in the absence of inducer thus minimizing the leaky expression and regulating the expression of several genes within a short time (Bocobza and Aharoni, 2014). Their use in plants has recently become widespread as described by Verhounig et al. (2010) who design a synthetic riboswitch from plastids to regulate growth and development of transgenic *N. tabacum* plants.This synthetic riboswitch caused significant induction in gene expression level even in the absence of ligand and without any leakage thus strengthening their growth and productivity. Since its inception, this riboswitch has been functionally implemented using a golden gate molecular suite in other plant species such as *Arabidopsis thaliana*, as well as in other model plants (Wachter, 2010) where the modified theophylline riboswitch under the control of transcriptional repressors resulted in increased expression of genes. However, its implementation remains elusive in other plant species for enhancing their growth and development under various abiotic stress conditions. Due to tight regulation, cellular independencies and low-price, theophylline dependent riboswitches are used in cyanobacterial systems. However, efforts are being diverted towards discovering new riboswitches sensing metabolites such as thymine pyrophosphate, flavin mononucleotide, queuosine, roseoflavin, cyclic dinucleotides (Breaker, 2011) nonetheless their genetic suitability needs to be further evaluated in model legume plants.

4.4.3. Small RNA (sRNA) mediated tools

Synthetic biology tools based on small RNAs (sRNA) have shown

their potential in efficiently controlling various cellular and biological processes via mediating post-transcriptional regulation by performing sequence-specific degradation of the endogenous transcript or translational inhibition (Xie et al., 2012). Besides, sRNA regulatory tools based synthetic sRNAs have shown their plausible role in enhancing the abiotic stress tolerance in plants as well as inimproving their growth and development under heavy metal stress conditions (Dai and Zhao, 2011). sRNAs mediated regulation of signal transduction pathways are of utmost importance in plants as they play a major regulatory role for their adaptability under extreme environments (Cabello et al., 2014). Furthermore, differential RNA (dRNA) sequencing study has also contemplated the ability of sRNA to modulate genome-wide regulation of target genes by regulating several non-coding RNAs (Xie et al., 2012). Moreover, sRNA regulatory tools can also be engineered strategically by removing non-essential genes or by targeting multiple pathways that may have a lethal effect on host cells using knocked down strategy (Axtell, 2013). sRNA has gained much recognition and to-date various sRNAs have been functionally characterized in plants but only a few have been exploited as a genetic tool (Axtell, 2013). Abiotic stress regulation by sRNA as a genetic tool for regulating translation of cis-repressive mRNA using a trans-activating RNA was first identified in *Arabidopsis* (Borges and Martienssen, 2015). Additionally, researchers have reported that several conserved and non-conserved sRNA are also involved in the regulation of abiotic stress tolerance in plants (Table 1). However, the expression of sRNA differs from species to species i.e. from domesticated plants to those that are adapted to suboptimal conditions (Axtell, 2013; Borges and Martienssen, 2015).

Researchers have successfully exploited sRNA tools for regulating various metabolic pathways in plants against heavy metal stress conditions (Ghosh et al., 2017). Several miRNA families like *miR397, miR408, miR857, miR398* have been shown to regulate Cu stress tolerance in most of the plants by regulating the expression of several enzymatic and non-enzymatic genes (Sunkar et al., 2012). Also, the upregulation of *miR95* in *Arabidopsis* plants has been shown to increase the tolerance of transgenic *Arabidopsis* plants to As and Cu stress compared to their wild counterparts (Liang et al., 2012). Simultaneously, Wang et al. (2019) also identified 18 new and 12 conserved miRNAs in *Paeonia ostia* where overexpression of these miRNA has been implicated in various crucial and multiple biological processes under heavy metal stress conditions. A study conducted by Yamasaki et al. (2009) has demonstrated that the presence GTAC motif in the promoter region of squamosa promoter binding protein-like 7 (*1SPL7*) is an utmost requirement of Cu paucity. Additionally, they also reported the activation of SPL7 TF is essential for regulating miR857 expression in improving various physiological and biochemical processes of *Arabidopsis* plants exposed to Cu stress. Fang et al. (2013) have identified and characterized both conserved and non-conserved miRNA such as *miR156, miR171* and *miR396a* in regulating growth and development of *Brassica napus* plants exposed to Cd stress. Similarly, *Medicago truncatula* plants exposed to Cd stress showed up-accumulation of *miR171, miR319* and *miR393* and down-regulation of *miR166* resulting in improved tolerance of *Medicago* plants by activating the expression of genes of enzymatic and non-enzymatic defense system (Zhou et al., 2008).

Besides, both *cis* and *trans*-acting sRNA can also be efficiently used to modulate gene expression that eventually can lead to improved signal transduction pathways thus enhancing abiotic stress tolerance (Liang et al., 2012). With the help of next-generation sequencing (NGS) technologies, deep RNA sequencing, high-resolution MALDI-TOF analysis and several *in-silico* based approaches it has now become possible to identify several sRNAs that can stimulate stress tolerance in those plants whose genomes are not completely sequenced (Kruszka et al., 2012). Therefore, to enhance the production of secondary metabolites, medicinal compounds as well as agricultural produce of under-utilized legumes application of sRNA mediated genetic tools can be most effective in the near future.

4.4.4. Application of reporter genes

Functional characterization using synthetic biology tools in terms of their expression, interaction and location can be achieved by utilizing a suitable reporter gene which allows easy detection of several biological circuits without having any adverse effect on the target organisms (Kumar et al., 2015b). The feasibility of reporter genes depends upon their fluorescent intensity and subcellular localization as detected by microscopic techniques. Among various reporter genes luciferase and fluorescent proteins are the most prevalent non-invasive reporters commonly used in the plant systems. In plants a number of reporter genes have been developed and are characterized as physical, chemical or metabolic sensors depending on the nature of input signals perceived by them (Rilling et al., 2019). Metabolic sensors comprised of dehalogenase gene from *Pseudomonas putida* capable of metabolizing 2,2 dichloropropionic acid (dalapon) a chlorinated aliphatic herbicide that permit the selection of transgenic plants (Caspi et al., 2014). Similarly, overexpression of peptide deformylase gene has been shown to confer resistance to *Arabidopsis* plants against herbicide actinonin (Houtz et al., 2010). The selectable marker genes *HSP101*is also involved in conferring wide range abiotic stress tolerance in plants and is also used as a reporter gene for the selection of transgenic *N. tabacum* plants against high temperature stress (Rosellini, 2012). The isopentenyl transferase gene of cytokinin biosynthetic pathway can also be used for the identification of transgenic plants having superior phenotype (Guo et al., 2010). Maize homeobox gene *KN1* which is also involved in cytokinin biosynthesis has been extensively used for the selection of transgenic plants having superior growth and physiology (Bolduc et al., 2012).

Additionally, reporter genes controlling mercury toxicity have been developed using modified *merA* gene from gram-negative transposon in *Arabidopsis thaliana* exhibiting enhanced Hg tolerance in transgenic *Arabidopsis* plants compared to wild form (Uraguchi et al., 2019). The mercury(II) reductase A (*merA)*promoter along with actin 2 (*ACT2)* promoter have the capacity to stimulate the transcription of stress-responsive protein complex thus efficiently conferring heavy metal stress tolerance in *Populus nigra* plants (Sakamoto et al., 2016). This *merA* promoter has been utilized in detecting toxic proteins by modulating catabolite repression thus down-regulating the expression of genes encoding toxic proteins. Similarly, reporter genes such as *MPR1* gene for L-proline analog resistance, nitrate reductase (*NiR* gene), phytoene desaturase (PDS), trehalose-6-phosphate synthase (*TPS1*), tryptophan decarboxylase (*TDC)* and xylose isomerase (*XylA*) controlling the expression of genes involved in metabolic pathways have been reported in several plant species (Rosellini, 2012). The expression of salt overly sensitive (*SOS*) responsive reporters under the influence of DREB TFs and *ABRC* promoter regulated the expression of *SOS1* gene thus conferring tolerance to plants exposed to heavy metals and salt stress conditions (Tang et al., 2010). Recently, researchers have developed ATP-binding cassette (ABC) transporter by using *A. thalianaWBC19* gene localized in the vacuole (Mentewab et al., 2014). This ATP-binding cassette (*ABC*) transporter gene have shown to confer resistance in *Arabidopsis* plants by facilitating the transport of kanamycin into the vacuole (Mentewab et al., 2014). In conclusion, all the above-mentioned reporter genes can be exploited for the development of improved and efficient reporter genes which could be valuable in facilitating gene transfer and promoting gene expression in several plant species under heavy metal stress conditions.

5. Improvement of heavy metal stress tolerance through CRISPR/Cas system

Clustered regulatory interspaced short palindromic repeats (CRISPR) CRISPR associated protein 9 (Cas9) systems are advanced RNA-guided genome editing systems identified first from *Streptococcus pyogenes* as part of an adaptive immune response against invading nucleic acid (Shan et al., 2013). The CRISPR/Cas and associated technologies have revolutionized the way genomes are being engineered in the present era.

The CRISPR/Cas systems (Fig. 3) include an array of flanking sequences termed as "spacer sequences" which upon transcription are converted to precursor CRISPR RNA (crRNA). Further, when this crRNA combines with transactivating crRNA (tracrRNA) forms mature crRNA/tracrRNA complex that is driven in the presence of Cas nuclease guided by spacer sequence and binds to protospacer-adjacent motif (PAM). The binding induces a double-strand break (DSB) in the desired DNA sequence and finally, this CRISPR edited DNA template is further exploited for the sequential alteration of genes of distinct tissues and organs (Feng et al., 2013). In eukaryotes, CRISPR/Cas associated technologies are currently being used for engineering genomes of several crop plants against various abiotic stresses whereas tailoring plants against heavy metal stress using CRISPR/Cas system is still in the exploratory phase (Belykh et al., 2019). This editing technology has significantly impacted the field of advanced molecular biology by allowing powerful, dynamic and efficient modification of template DNA sequences (Tang et al., 2017).

The implementation of the CRISPR/Cas system in plant heavy metal stress tolerance is a promising venture to increase the phytoremediation ability of plants (Belykh et al., 2019). Additionally, with the availability of the complete genomic sequences of most of the model phytoremediators such as*Arabidopsis halleri*, *Pteris vittata*, *Noccaea caerulescens*, *Hirschfeldia incana* and *Brassica juncea* could serve as a platform for the identification and characterization of important genes involved in the enhanced metal stress tolerance (Kumar and Trivedi, 2018). Furthermore, CRISPR/Cas systems can also stimulate various other processes such as phytoaccumulation, phytodegradation, phytostabization and rhizofiltration (Pérez-Palacios et al., 2017). It can also facilitate the development of transgenic plants containing desired set of instructions more efficiently as compared to other genome editing tools such as ZFNs and TALENs (Shan et al., 2013). One of the areas where CRISPR/Cas system could be more effective for stimulating phytoremediation is by targeting the expression of those genes which are involved in the metal transport such as MATE, YSL, CDF and ZIP family proteins as well as those genes that act as metal ligands such as metallothioneins and phytochelatins (Basu et al., 2018). Several studies have reported that overexpression of metallothioneins encoding genes (*MT1* and *MT2*), *APS*, *SMT*, *NRAMP* and plethora of other metal responsive (mentioned in the above section) have resulted in the increased tolerance of various plants against heavy metal stress (Basu et al., 2018). Therefore, all the above-mentioned genes along with other important genes can be rewired for stimulating heavy metal stress tolerance in plants using CRISPR/Cas system (Table 3). Recently, this technology was used in the generation of transgenic *O. sativa* lines exhibiting strong tolerance to Cs stress by knocking-down the expression of *OsHAK1* gene leading to inactivation of K^+ transporter thereby decreasing the Cs uptake (Nieves-Cordones et al., 2017). Tang et al. (2017) also used CRISPR/Cas9 system to create knockout *O. sativa* lines by mutating the expression of *OsNRAMP5* gene which resulted in decrease uptake of Cd-accumulating indica *O. sativa* without affecting its yield. Recently, Yang et al. (2019) also employed CRISPR/Cas9 system to increase the tolerance of japonica *O. sativa*exposed to Cd stress by creating *OsNRAMP5* knockout lines (Table 3).

The CRISPR/Cas system still has its limitation in plants and the major constraint of CRISPR/Cas mediated transformation in plants is the higher discrepancies among transformed colonies for desired genomic alteration. Previous reports on plant transformation have indicated that only 20–50% of the CRISPR-transformed colonies were having the desired mutation (Basu et al., 2018). Over-expression of Cas9 construct has been shown to have a lethal effect in most of the plant species which may be due to the toxicity of Cas9 protein which may affect

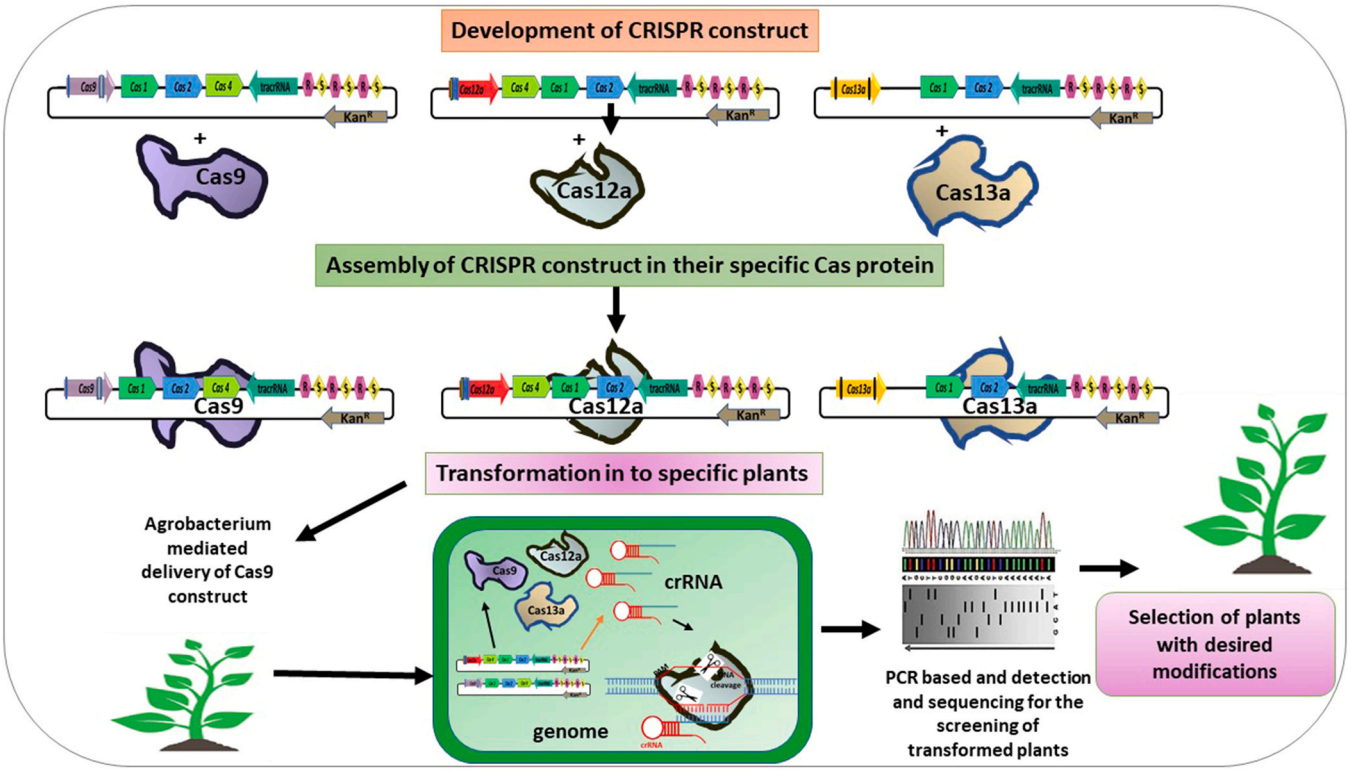

Fig. 3. Overview of CRISPR/Cas system for engineering plants against heavy metal stress. The figure represents the arrangement of loci and domains of CRISPR protein systems. Among Cas9, Cas12a and Cas13a, only Cas9 function in the presence of tans-activating CRISPR RNA (tracrRNA) whereas Cas12a and Cas13a function in the absence of tracrRNA. The CRISPR/Cas system uses single plasmid containing directs and spacer sequences under tight regulation of lac promoter and crRNA encoding genes which induces the transient expression of respective Cas proteins. The CRISPR/Cas construct is then transformed into specific plants for cleavage of the target sequence. Immediately after the transformation, double stranded break is introduced which is further repaired by homologous recombination mediated by template plasmid. The selection of respective transconjugants is done through PCR or by sequencing of transformed plants with desired traits.

Table 3
Application of CRISPR-Cas system-based synthetic tools for engineering plants against heavy metal stress.

Cyanobacterial species	Engineering approach	Target gene	Cas protein used	Vectors and selection markers used	Expression type and promoter used	Trait engineered	References
Oryza sativa	Gene knockout	*OsNramp5*	dCas9	Cas9 binary vector, Hygromycin	Suppressed expression, OsU6 and OsU3	Knockout of OsNramp5 resulted in decreased accumulation of Cd	Tang et al. (2017)
Arabidopsis thaliana	Gene editing	*AtPDF2.6*	dCas9	binary vector pCAMBIA1300, hygromycin	Increased expression, ProAtPDF2.6	AtPDF2.6 detoxifies cytoplasmic Cd via chelation and thereby enhances Cd tolerance in Arabidopsis	Luo et al. (2019)
Catharanthus roseus	Multiple gene repression	*CrRLK1L*	dCas9	GreenGate entry vectors, Hygromycin	Inducible expression, ubiquitin4–2	CRISPR mediated repression of genes resulted in improved growth in response to metal ions	Richter et al. (2018)
Oryza sativa	Gene knockout	*OsATX1*	dCas9	GreenGate entry vectors, Hygromycin	Heterologous expression, OsATX1Pro:GUS	OsATX1 overexpression reduced root Cu concentrations but increased Cu accumulation in the shoots leading to improve growth	Zhang et al. (2018)
Oryza sativa	Gene knockout	*OsHAK1*	Cas9	pHUbiCas9, pYPGE15, Hygromycin	Transient expression, crRNA	Targeted suppression of *K+ transporter OsHAK1* resulted in low accumulation of Cs	Nieves-Cordones et al. (2017)
Oryza sativa	Gene knockout	*OsARM1*	Cas9	pYLCRISPR/gRNA vector Hygromycin	Over expression, OsARM1 promoter	OsARM1 was involved in the regulation of As tolerance in rice, possibly by modulating the uptake and root-to-shoot translocation	Wang et al. (2017a)

transformation efficiency or limit the functionality of spacers used for targeting the desired genomic region (Shan et al., 2013). However, the probable mechanism behind the lethality of Cas9 remains elusive (Belykh et al., 2019). Meanwhile, efforts are constantly being made to develop a target-based inducible system to overcome the fatality of present Cas9 system in plants. This include bypassing Cas9 with Cpf1 system (CRISPR from *Prevotella* and *Francisella* 1) that minimizes off-target transformation thus increasing the efficiency of transformation. The Cpf1 does this by employing constitutive promoters/riboswitches that accurately regulate the transcription of small guide RNAs (sgRNAs) with minimum leakage (Chen et al., 2019). This CRISPR/Cpf1 system has been successfully used in various plant species for generating knock-ins, knock-outs and introducing point mutations without having any destructive effect on hosts (Xu et al., 2017). Recently, this system has also been exploited to upregulation of various downstream genes of metabolic pathways by activating their transcription using artificial TFs (Xu et al., 2019).

6. Conclusion and future perspective

Rapidly changing environmental conditions due to enhanced anthropogenic activities affect the agricultural productivity of plants that in turn influences the demand-supply gap of the global population. Therefore, it has become necessary to engineer crop plants i.e. targeting genes involved in signaling and regulatory pathways through advance biotechnological tools. Genome editing through site-specific nucleases can be used to improve abiotic stress tolerance including heavy metal stress by targeting specific genes. In this context, the CRISPR/Cas system has emerged as a ground-breaking technology for genome editing in plants. The CRISPR/Cas9 system has been widely employed for tailoring plant species against abiotic/biotic stresses however; their implementation in enhancing heavy metal tolerance is still limited. However, some studies have documented that the genetically modified (GM) crops are causing allergies, inflammation and are harmful for humans and animals. Nonetheless, these studies when reassessed for their reliability showed significant flaws in their design, execution and analysis. Foods derived from GM crops have been extensively consumed over past few decades in developed countries with no ill effects. The GM crops are being used to develop recombinant DNA medicines, antibodies, biofuels, and plastics and in future can help alleviate the challenges of food and medicine supply. Furthermore, researchers working in the field of plant science community are keenly developing synthetic biology tools such as synthetic promoters, riboswitches and other gene regulatory tools for effective genetic manipulation of plants against heavy metal stresses. Overall, genetic manipulations related to heavy metal responsive genes, transcription factors and metal proteins/ligands have shown tremendous outcome in increasing the plant tolerance to heavy metal stress as well as their phytoremediation ability. However, their full potential needs further exploration by designing futuristic experiments using multi-disciplinary approach i.e. both system and synthetic biology approaches to develop improved crop varieties with enhanced tolerance to a wide range of abiotic stresses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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