

Review

Cadmium tolerance and hyperaccumulation in plants – A proteomic perspective of phytoremediation

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ABSTRACT

Cadmium (Cd) is a major environmental pollutant and poses a risk of transfer into the food chain through contaminated plants. Mechanisms underlying Cd tolerance and hyperaccumulation in plants are not fully understood. Proteomics-based approaches facilitate an in-depth understanding of plant responses to Cd stress at the systemic level by identifying Cd-inducible differentially abundant proteins (DAPs). In this review, we summarize studies related to proteomic changes associated with Cd-tolerance mechanisms in Cd-tolerant crops and Cd-hyperaccumulating plants, especially the similarities and differences across plant species. The enhanced DAPs identified through proteomic studies can be potential targets for developing Cd-hyperaccumulators to remediate Cd-contaminated environments and Cd-tolerant crops with low Cd content in the edible organs. This is of great significance for ensuring the food security of an exponentially growing global population. Finally, we discuss the methodological drawbacks in current proteomic studies and propose that better protocols and advanced techniques should be utilized to further strengthen the reliability and applicability of future Cd-stress-related studies in plants. This review provides insights into the improvement of phytoremediation efficiency and an in-depth study of the molecular mechanisms of Cd enrichment in plants.

1. Introduction

Cadmium (Cd) is classified as a priority heavy metal pollutant because of its high toxicity, high carcinogenicity, and widespread contamination of the environment. Cd enters the human food chain mainly through the direct consumption of Cd-contaminated plants, shellfish, or meat sourced from animals raised on contaminated plant-origin feed (Verbruggen et al., 2009; Chen et al., 2020). Natural and man-made manufacturing processes are both responsible for the release of more than 10,000 tons (9072 metric tons) of Cd into the environment,

where it can seep into drinking water supplies and contaminate soil in agricultural fields (Troutner, 2022). High Cd levels in agricultural soil are primarily due to the overuse of phosphate fertilizer, seepage of industrial effluents, and urban sewage sludge (Munira et al., 2016). Several electrochemical sensors can accurately detect the presence of heavy metals such as Cd and cesium in the environment (Rezayi et al., 2011, 2012; Kassim et al., 2011; Ahmadzadeh et al., 2015; Solomonea et al., 2022). For example, a portable optical fiber surface plasmon resonance sensor was used to detect trace amounts of Cd²⁺ (Solomonea et al., 2022). Cd content in phosphate fertilizers can be as high as 200 µg

Abbreviation: CAL1, Cd accumulation in leaf 1; Cd, cadmium; CHS, chalcone synthase; CR, Calabash Rouge; DAPs, differential abundance proteins; DW, dry weight; GS, glutamine synthetase; GST, glutathione S-transferase; HMA3, Heavy Metal ATPases3; HP, hyperaccumulating population; HSPs, heat shock proteins; iTRAQ, isobaric tags for relative and absolute quantification; MT, metallothionein; MTP, metal transport/tolerance proteins; NHP, non-hyperaccumulating population; N/S, nitrogen/sulfur; PC, phytochelatin; PR, Pusa Ruby; PTMs, post-translational modifications; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SILAC, stable isotope labeling by amino acids; SOD, superoxide dismutase; TCA, citric acid; XGA, xylogalacturonans; 2-DE, two-dimensional polyacrylamide gel electrophoresis.

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g^{-1} (Moreno et al., 1999), and be up to $815 \mu\text{g g}^{-1}$ in sewage sludge (Qadir et al., 2000). In China alone, nearly 20 million hectares of arable land are contaminated with Cd and other heavy metals, resulting in the production of 12 million tons of contaminated grains annually (Luo et al., 2018). Therefore, remediation of Cd-contaminated arable land has become a global priority.

As a result of long-term natural selection and adaptation, some plants have developed high Cd-tolerance, and their shoots can accumulate Cd at concentrations above even $100 \mu\text{g g}^{-1}$ dry weight (DW), which is regarded as the threshold value for Cd-hyperaccumulators (Clemens, 2001). For example, *Arabidopsis halleri* and *Sedum plumbizincicola* can accumulate Cd above 1000 and 7000 $\mu\text{g g}^{-1}$ DW, respectively (Zhao et al., 2006; Liu et al., 2017). Such hyperaccumulators possess exquisite Cd-detoxification mechanisms, without demonstrating any effects of the toxicity emanating from the accumulation of massive levels of Cd. Only a limited number of Cd-hyperaccumulating plant species have been identified (Isaure et al., 2015), whereas a vast majority of them are susceptible to Cd-toxicity due to their genetic background.

Phytoremediation of Cd-contaminated soil and water through using Cd-hyperaccumulators is an effective, low-cost, and sustainable method to prevent threats to ecological safety, agricultural production, and human health from Cd-toxicity (Kanwar et al., 2020). The methods such as heat, extraction, and microbial treatments; synthesis of nano-materials; and compression landfill, to deal with the disposal of plant biomass containing heavy metals, after utilization of plants for phytoremediation, were recently reviewed (Liu and Tran, 2021). For effective phytoremediation, the selected plants should ideally be able to both tolerate and accumulate high levels of Cd, while maintaining rapid growth rates to translocate the excessive Cd from the soil into the easily-harvestable aerial parts. Moreover, the ideal phytoremediation plants should have a large biomass production capacity that suffers little adverse effects of long-term Cd-stress (He, 2013; Gutsch et al., 2018a). However, large-scale application of phytoremediation is restricted by the availability of plants from diverse species backgrounds.

Alternatively, many crops have demonstrated phytoremediation potentials, exhibiting significant differences in Cd-tolerance and accumulation ability across cultivars, providing varied options for Cd-phytoremediation (Fig. 1). E.g., *Medicago sativa* (alfalfa) and *Linum usitatissimum* (flax) can maintain sufficient biomass and high growth rates under Cd-stress (Gutsch et al., 2018a; Rehman et al., 2021). Numerous studies have revealed the important roles of specific genes/proteins in Cd-tolerance in plants (Krämer, 2010; Migocka et al., 2015a, 2015b; Liu et al., 2017; Zhao et al., 2019), but the underlying mechanisms of Cd-tolerance and accumulation in Cd-hyperaccumulators are not yet fully clarified. Thus, a better understanding of Cd-tolerance and accumulation mechanisms in plants is essential for developing novel Cd-hyperaccumulators.

The proteome represents the total set of proteins synthesized by an individual cell of an organism or a type of tissue or an organ system at a specific time point or state (Vistain and Tay, 2021). Proteomics-based research has provided revealing insights into Cd tolerance and accumulation processes in plants (Kieffer et al., 2009a; Singh et al., 2016; Winter et al., 2019; Dai et al., 2020). Several articles have recently reviewed proteomic analyses of plant response to heavy metals (Farinati et al., 2009; Dalcorso et al., 2013; Visioli and Marmiroli, 2013; Ahmad et al., 2019; Hussain et al., 2022), but comprehensive reviews on proteomic studies in Cd-hyperaccumulating plants are lacking, as of date. This review focuses on the proteome level changes in Cd-hyperaccumulating plants and Cd-tolerant crops under Cd stress and their potential application in phytoremediation of Cd-contaminated soil and water (Fig. 1). The promising use of Cd-inducible DAPs as potential targets for breeding or genetically engineering Cd-hyperaccumulators and Cd-tolerant crops are also highlighted.

Cd-tolerant and Cd hyperaccumulative plants or crops



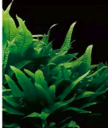












In water		In soil	
	Sargassum fusiforme a brown algae, grows along rocky coastlines, high Cd absorption capability		Water hyacinth (<i>Eichhornia crassipes</i>) floating, perennial, fast-growing aquatic plant, accumulating > 10,000 $\mu\text{g Cd/g}$ in root
	Java fern (<i>Microsorium pteropus</i>) water fern from Asia, 15-30 cm tall, accumulating > 4,000 $\mu\text{g Cd/g}$ in root and leaf		Indian mustard (<i>Brassica juncea</i>) an annual herb, 1 m or more tall, accumulating > 400 $\mu\text{g Cd/g}$ in leaf
	Sunflower (<i>Helianthus annuus</i>) an annual crop, 1-4.5 m tall, accumulating > 900 $\mu\text{g Cd/g}$ in stem and leaf		Alfalfa (<i>Medicago sativa</i>) an herbaceous perennial legume, used primarily as a feedcrop, high Cd absorption capability in shoot
	Flax (<i>Linum usitatissimum</i>) an annual fiber plant, high biomass and developed root system, high Cd tolerance and accumulation capabilities		Poplar (<i>Populus</i>) a tall, fast-growing dioecious plant, with developed root system, high Cd accumulation capability
	Crassulaceae (<i>Sedum alfredii Hance</i>) low biomass, asexual propagation, accumulating > 11,000 $\mu\text{g Cd/g}$ in leaf		Amaranth (<i>Amaranthus gangeticus</i>) a vegetable crop, accumulating > 100 $\mu\text{g Cd/g}$ in shoot
	American pokeweed (<i>Phytolacca americana</i>) a fast-growing plant with high biomass, accumulating > 240 $\mu\text{g Cd/g}$ Cd in leaf		Rice (<i>Oryza sativa</i>) an annual grass crop, major staple food, some cultivars have high Cd tolerance capability
	Tobacco (<i>Nicotiana tabacum</i>) an important cash crop, high Cd absorption capability in leaf		Soybean (<i>Glycine max</i>) an annual legume, 2-6 m tall, some cultivars have high Cd tolerance capability
	Chinese turnip (<i>Brassica rapa var. rapa</i>) a hardy biennial vegetable, has high Cd tolerance and accumulation capabilities in leaf		

Fig. 1. The representative plants with application potentials in phytoremediation of Cd-polluted environments. The Cd-inducible protein changes in these plants have been compared in Cd-contrasting accumulation genotypes.

Table 1
Representative proteomic studies conducted in Cd hyperaccumulators and contrasting Cd-accumulation plants exposed to Cd toxicity.

Material	Cd stress	Approach	Main results	Reference
<i>Chlorella sorokiniana</i>	250 μM of Cd^{2+} , 40 h, hydroponics	Nano-LC MS/MS	Photosynthesis and oxidative phosphorylation decreased, whereas photorespiration increased.	León-Vaz et al. (2021)
<i>Chlamydomonas reinhardtii</i>	150 μM Cd^{2+} , 4–5 d, hydroponics	2DE-MS/MS	Decrease of RuBisCO and other enzymes involved in photosynthesis; increase of enzymes related to antioxidative stress.	Gillet et al. (2006)
<i>Microsorium pteropus</i>	100, 250 and 500 μM Cd^{2+} , 7 d, hydroponics	2DE-MS/MS	Different proteomic responses in resistance to Cd stress between root and leaf.	Lan et al. (2018)
Turnip leaves	5 and 25 μM	Isobaric tags (IBT), MS/MS	DAPs were enriched on the glutathione metabolism pathway, involved in Cd detoxification.	Li et al. (2021)
Barley leaves	29 and 200 μM Cd^{2+} , 7 d, hydroponics	iTRAQ-MS/MS	Using isolated tonoplast from leaf mesophyll, several vacuolar transporters were identified in higher abundance after Cd exposure. They have a specific function in Cd^{2+} transport into the vacuole and play an important role in Cd^{2+} detoxification of barley leaf mesophyll cells.	Schneider et al. (2009)
Soybean cultivars with contrasting Cd-accumulation	100 μM Cd^{2+} , 3 and 14 d, hydroponics	2DE-MS/MS	In roots of low Cd-accumulating cultivar, NADP-dependent alkenal double bond reductase P1 were more abundant. In high Cd-accumulating cultivar, proteins associated with Cd-chelating pathways and lignin biosynthesis were increased in roots, enzymes involved in glycolysis, and those in TCA cycle and photosynthesis increased in leaf.	Hossain et al. (2012); Ahsan et al. (2012)
<i>Medicago sativa</i>	10 mg Cd/kg soil, ca. 4 month	2D-DIGE, MS/MS	Most increased DAPs (e.g. pectin methylesterase) upon long-term Cd exposure, involved in cell wall remodeling and lignification, defence response and carbohydrate metabolism	Gutsch et al. (2018a, b); Gutsch et al. (2019)
Wheat varieties with contrasting Cd tolerance	50 μM Cd^{2+} , 24 h	LC-MS/MS	DAPs in roots of two varieties were mostly related to DNA replication and repair, protein metabolism, and the GSH metabolism pathway.	Jian et al. (2020)
Tomato varieties with contrasting Cd tolerance	35 μM Cd^{2+} , 4 d	LC-MS/MS	DAPs were mainly associated to cell wall, redox, and stress responses, with increased abundance of intracellular components in Cd-sensitive variety enhanced and increased abundance of extracellular and envelope proteins in Cd-tolerant variety. Cd-responsive phosphoproteins were involved in Cd signaling and Cd stress tolerance in leaves.	Borges et al. (2019); Marques et al. (2021)
<i>Populus spp.</i>	20 μM Cd^{2+} , 48 h and 14 d, hydroponics	2DE, LC-MS/MS	The number of DAPs was more after 48 h stress than 14 d stress. Increase of stress response; decrease of carbohydrate metabolism both short- and long-term treatments.	Marmioli et al. (2013)
<i>Sedum plumbizincicola</i>	400 μM Cd^{2+} , 1 d and 4 d, hydroponics	TMT labeling, LC-MS/MS	Plasma membrane proteins were isolated from root, stem and leaf tissues. Among 352 Cd-inducible transport proteins, up-regulated transport proteins increased with stress time, especially in roots.	Zhu et al. (2022)

2. Proteomic analysis of typical Cd-hyperaccumulators and Cd-tolerant crops

2.1. Algae and hydrophytes

In aquatic environments, Cd-contamination easily spreads with water currents, resulting in difficulties in the remediation of Cd-polluted water (Singh et al., 2016). A majority of studies on the phytoremediation of Cd-contamination have thus far focused on utilizing terrestrial plants. Therefore, it is essential to identify microalgae and aquatic plants with Cd-hyperaccumulation traits for application in the remediation of Cd-contaminated water.

Algae are single- or multicellular organisms without differentiated organs like roots, stems, or leaves. They are thus different from terrestrial plants, which are exposed to Cd-toxicity mainly through the roots. The morphology, ultrastructure, and metabolic pathways of the whole algal cell are susceptible to Cd-toxicity in water (León-Vaz et al., 2021). Algal cells deploy various mechanisms, including biosorption, bio-accumulation, compartmentalization, and biotransformation or metal detoxification to minimize Cd toxicity (Gillet et al., 2006; Mota et al., 2015; León-Vaz et al., 2021; Wang et al., 2021a, 2021b). Microalgae can use biotransformation as one of the self-protection mechanisms to convert heavy metals into non-toxic compounds through bonding with chelating agents like organic acids, peptides, enzymes, and thiol-containing molecules. These non-toxic compounds are then either accumulated inside or extruded from the cell using efflux transporters (Wang et al., 2021a, 2021b). Proteomic analyses of algae exposed to high Cd stress have identified diverse Cd-inducible DAPs (Table 1). Cd-affected cellular processes in algae such as photosynthesis, oxidative phosphorylation, the citric acid cycle (TCA cycle), and ribosomal

proteins synthesis were significantly inhibited, whereas synthesis of antioxidant enzymes and heat shock proteins (HSPs), photorespiration, gluconeogenesis, starch catabolism, and nitrogen/sulfur (N/S) assimilation was significantly enhanced (Gillet et al., 2006; Mota et al., 2015; León-Vaz et al., 2021) (Table 1). In the multicellular alga *Sargassum fusiforme*, the majority of DAPs to be reduced in abundance were metabolic enzymes involved in carbohydrate and energy metabolism, but the alga may adapt to prolonged Cd-stress via increased glycolysis and TCA cycles to supply the requisite amounts of energy required for survival (Zhang et al., 2015). Microalgae, especially *Chlamydomonas* and *Chlorella*, have emerged as viable options for the phytoremediation of Cd-contaminated water due to their rapid reproduction rate and unique adaptation mechanisms to high-Cd-stress. It is necessary to then identify the candidate genes and proteins that can be used for strain engineering of microalgae to improve their phytoremediation efficiency.

Microsorium pteropus is an aquatic fern with a capacity for Cd-hyperaccumulation (Fig. 1) (Lan et al., 2018). After seven days of exposure to Cd-stress, eight and 20 DAPs were identified in the roots and leaves of *M. pteropus*, respectively through 2-DE (two-dimensional polyacrylamide gel electrophoresis)-based proteomic approach (Lan et al., 2018). In *Populus yunnanensis* (poplar), root and leaf tissues adopted different Cd-resistance mechanisms: the roots resisted Cd-toxicity by mainly upregulating antioxidants and regulating energy metabolism, whereas leaves by mainly maintaining photosynthesis rates and regulation of cellular metabolism (Yang et al., 2015a).

The hydrophytes *Eichhornia crassipes* (water hyacinth) and *Pistia stratiotes* are widely used in the phytoremediation of sewage to control eutrophication and heavy metal pollution (Wang et al., 2012). *E. crassipes* exhibited higher tolerance to high-Cd-stress than *P. stratiotes* at the morphological and physiological levels (Fig. 1). A comparative

proteomic analysis of the two genera exposed to Cd-stress revealed that *E. crassipes* was more tolerant to Cd-toxicity due to upregulation in the synthesis of analogous proteins to compensate for the downregulation of certain proteins involved in cellular activities to maintain their corresponding functions (Li et al., 2015). In particular, several compounds involved in stress-resistance, such as the amino acid proline, HSPs, and processes such as post-translational modifications (PTMs) of proteins were involved in the protection and functional restoration of proteins in *E. crassipes*. Moreover, antioxidant enzymes played important roles in the scavenging of excess reactive oxygen species (ROS) to minimize Cd-induced oxidative stress (Li et al., 2015). These studies demonstrate that *E. crassipes* could better maintain cellular metabolism than *P. stratiotes* under Cd-stress, thus making it a more promising option for the phytoremediation of Cd-contaminated water.

2.2. Brassicaceae plants

The Brassicaceae family includes some important vegetables and oil-containing plants cultivated worldwide. Several *Brassica* species and landraces exhibit Cd-hyperaccumulation traits that may have evolved as a result of intraspecific variations.

Brassica rapa var. *rapa* (Chinese turnip) has strong Cd-tolerance and accumulation capabilities (Fig. 1). In a particular Cd-hyperaccumulating landrace in turnip, the transcriptomic analysis revealed that more than 80 % of the upregulated genes in leaves were mainly associated with pathways such as sulfur metabolism, flavonoid biosynthesis, and glucosinolate biosynthesis involved in the detoxification mechanism of Cd (Li et al., 2021). Proteomic analysis of turnip leaves demonstrated that the elevated DAPs were mainly involved in sulfur assimilation, glutathione metabolism, and modification of protein acetylation, which seemed to be the key pathways in Cd-tolerance and detoxification (Li et al., 2021). Three metal transport/tolerance proteins (MTP) and a heavy metal ATPase (HMA) 2.1 were found to be distinctly responsible for the Cd-accumulation capacity and metal transition processes in turnip leaves, of which *MTP1* gene encoding for a Zn^{2+}/H^{+} antiporter was highly expressed as observed in Zn-hyperaccumulators (Shahzad et al., 2010).

Brassica juncea (Indian mustard) can accumulate Cd to levels $> 400 \mu\text{g g}^{-1}$ DW in leaves over 24 days of exposure, despite some deleterious effects on plant growth (Haag-Kerwer et al., 1999). In *B. juncea* exposed to $250 \mu\text{M Cd}^{2+}$ for three days, an array of proteins involved in sulfur assimilation, redox homeostasis, and xenobiotic detoxification, especially O-acetylserine sulfhydrylase, glutathione S-transferase (GST), 2-nitropropane dioxygenase, peptide methionine sulfoxide reductase, and glutathione-conjugate membrane transporter were accumulated in abundance in roots (Table 1, Fig. 1). These DAPs could potentially be involved in Cd-tolerance and hyperaccumulation in *B. juncea* (Alvarez et al., 2009). Another proteomic analysis revealed Cd-induced alterations in photosystem components, sulfur metabolism, energy metabolic pathways (e.g., Calvin cycle and photorespiration), and antioxidant responses [especially glutathione and phytochelatin (PC) homeostasis] in *B. juncea*. Moreover, levels of phosphoglycolate, a byproduct of photorespiration, were remarkably elevated in leaves, implying that *B. juncea* relied on photorespiration to cope with Cd-induced imbalances in stomatal conductance and deregulation of CO_2 homeostasis (D'Alessandro et al., 2013).

In those accessions of *Thlaspi caerulescens* which are recognized as Cd/Zn-hyperaccumulators, the Cd-induced DAPs were mainly involved in core metabolic functions (e.g., photosynthesis, N/S assimilation, and carbohydrate metabolism), signaling pathways, and regulatory functions (Tuomainen et al., 2006). Differentially expressed genes that were involved in the homeostasis of specific metal ions in *T. caerulescens* exhibited significant variations in comparison with those in a non-hyperaccumulator, *A. thaliana* (van de Mortel et al., 2008). Similarly, in the metal hyperaccumulator *Arabis paniculata* Franch, 16 Cd-responsive DAPs mainly involved in antioxidative/xenobiotic

defense, cellular redox homeostasis, and Cd-translocation in the shoots were identified (Zeng et al., 2011). To summarize, *Brassicaceae* plants initiate various pathways in response to high-Cd-stress including antioxidant systems, osmotic adjustment, chelating effects, etc. (Isaure et al., 2015). The relevant DAPs may be of great interest as candidate genes for the molecular breeding of Cd-hypoaccumulating plants as a source of food or Cd-hyperaccumulating plants for the phytoremediation of Cd-contaminated soils.

2.3. Legume plants

Medicago sativa (alfalfa) a model legume plant is also an important forage crop globally. It can accumulate Cd and other heavy metals, with the highest accumulation of Cd in roots, followed by stems, and the least in leaves (Gutsch et al., 2018a). To explore the adaptive mechanisms of *M. sativa* to a four-month-long period of exposure to high-Cd-stress (10 mg Cd kg^{-1} soil, mimicking actual Cd-contaminated soil), the proteome profile changes in cell walls of leaf, root, and stem tissues were analyzed using a gel-based approach, through which 212 and 179 Cd-induced DAPs were identified in leaves and stem, respectively (Gutsch et al., 2018a, 2018b). Except at the early stages of growth, mature alfalfa plants exposed to long-term Cd-stress exhibited no significant phenotypic and biomass differences when compared to control plants. The bioconcentration and translocation factors of Cd in alfalfa were 1.60 and 1.17 respectively, indicating that it is one of the most suitable species for extraction of Cd from contaminated soil (Zhang and Ji, 2019). Alfalfa transports Cd from the roots to the aboveground organs which are then collected and treated uniformly, resulting in a decrease in Cd content in the soil (Suman et al., 2018). Thus, due to its fast growth rate, large biomass, and high Cd-tolerance, alfalfa may be selected for further improvement by genetic engineering to transform into a Cd-hyperaccumulator and then used for phytoremediation of Cd-contaminated pasture lands.

Glycine max (soybean) is widely cultivated for the high oil and protein content of its seeds. In recent years, an increased Cd content in soybean seeds was observed worldwide, due to elevated levels of soil contamination and low soil pH (Zhang et al., 2021). To study Cd-stress responses in soybean, cells obtained from suspension cell culture were exposed to $1\text{--}10 \mu\text{M Cd}$ (mimicking contaminated soil) for 1–3 days (Sobkowiak and Deckert, 2006). Cd-induced protein changes were analyzed using one-dimensional electrophoresis and ^{35}S -labelling autoradiography. The significantly elevated, Cd-induced DAPs mainly included superoxide dismutase (SOD), histone H2B, chalcone synthase (CHS), and GST, their accumulation levels were Cd dose- and time-dependent. These proteins are recognized to function in antioxidant systems (SOD), detoxification systems (GST), DNA damage repair (H2B), and stress tolerance (CHS) (e.g., Cd stress) (Dao et al., 2011).

Soybean is tolerant to low-Cd stress with limited loss in yield, but high-Cd stress significantly affects plant growth and yield (Ikhajagbe et al., 2021). Considerable genetic variations among soybean cultivars concerning Cd-uptake, accumulation, and translocation to the aerial parts were observed (Arao et al., 2003). E.g., the cultivars 'Enrei' and 'Fukuyutaka' are low-Cd-accumulators, whereas 'Harosoy' is a high-Cd-accumulator which is more efficient in translocating Cd from root to shoot (Ahsan et al., 2012; Hossain et al., 2012). To identify organ-specific, Cd-responsive proteins involved in Cd translocation and detoxification in soybean, comparative proteomic analyses between the contrasting Cd-accumulating cultivars exposed to $100 \mu\text{M Cd}$ were performed (Hossain et al., 2012). NADP-dependent alkenal double bond reductase P1 accumulated conspicuously in the roots of the low-Cd-accumulating cultivar. However, leaves of both cultivars shared some common strategies to cope with Cd-stress such as enhanced levels of enzymes involved in glycolysis, TCA cycle, and photosynthesis to increase energy production; elevation in levels of antioxidant enzymes to scavenge the excess ROS and increased glutathione and PC concentrations to detoxify cytosolic Cd^{2+} (Hossain et al., 2012). Moreover, the

synthesis of HSPs was enhanced in the high-Cd-accumulating cultivar as an additional Cd-detoxification mechanism. Cd-induced protein changes in the microsomes from roots of two contrasting Cd-accumulating soybean cultivars were compared; amino acids and proteins associated with Cd-chelation pathway and lignin biosynthesis were elevated by Cd-stress in the roots of the low Cd-accumulating cultivar (Ahsan et al., 2012). These Cd-chelating agents bind to excess free Cd²⁺, resulting in a reduced Cd²⁺ translocation from root to shoot through an enhanced Cd-chelation pathway and xylem lignification.

2.4. Cereal plants

Cereal crops such as wheat, rice, and maize are major sources of staple foods for a vast majority of the global population. Cd contamination in soils leads to its accumulation in cereal grains posing a severe threat to human health and food security (Sebastian and Prasad, 2019). There were significant differences in Cd-tolerance and accumulation abilities among the available cereal cultivars (Sun et al., 2013; Jian et al., 2020). Some Cd-tolerant varieties of crops can accumulate only a certain limited amount of Cd. For example, the wheat cv. 'Xinong 20' can accumulate up to 18 mg Cd kg⁻¹ DW under cultivation on 50 mg Cd kg⁻¹ soil (Zhang et al., 2019). Owing to their rapid growth rate and large biomass, crops such as wheat and rice may be genetically improved for the phytoremediation of Cd-polluted agricultural soils. Therefore, comparative proteomic studies between the cultivars with contrasting Cd-tolerance traits help understand Cd response mechanisms in cereals and in developing Cd-tolerant cultivars with low Cd content in grains through directional selection and molecular breeding.

M1019 is a Cd-tolerant inbred line, whereas 'Xinong 20' is a Cd-sensitive cultivar of wheat. After exposure of roots to 50 µM Cd²⁺ for 24 h, M1019 accumulated Cd mainly in straws (enriched in cell walls), whereas 'Xinong 20' accumulated it mainly in the leaves (Table 1). Moreover, the Cd content in grains of M1019 was significantly lower than that of 'Xinong 20'. Comparative proteomic analyses revealed that the elevated DAPs, such as GST (a key enzyme in glutathione synthesis) and HSPs may be related to enhanced Cd-tolerance in M1019 (Jian et al., 2020). As observed with the other Cd-tolerant plants discussed above, M1019 also utilized glutathione metabolism and sulfur metabolism (cysteine and methionine) for detoxification. A comparison of the proteomic profiles from two barley genotypes differing in grain Cd-levels indicated that the enhanced DAPs in the grains of the high Cd-accumulation genotype mainly included stress proteins, storage proteins, and those involved in carbohydrate metabolism (Sun et al., 2013). Moreover, protease inhibitors such as serpins (serine protease inhibitors) were abundant in high Cd-accumulation grains, implying a role of serpins in Cd-accumulation, transport, and protection of storage proteins from Cd-toxicity.

Rice is very sensitive to Cd-stress, exhibiting a delayed growth pattern and yield loss due to reduced photosynthesis. Cd concentration in rice grains was found to be as high as 1–2 mg kg⁻¹ DW in some rice-growing areas of China, much higher than the national average of 0.2 mg kg⁻¹ DW (Cheng et al., 2006; Zhu et al., 2022). Therefore, remediation of soil used for paddy cultivation and reduction of Cd-accumulation in rice grains are of high priority. The changes in root and leaf proteomes under short-term exposure to Cd were compared; in roots, most DAPs were oxidative stress-related, of which glutathione reductase (GR, catalyzing the reduction of glutathione disulfide to glutathione), GST, and endo-1, 3-beta-glucanase (pathogenesis-related proteins) were significantly elevated. Meanwhile, a dramatic decrease in the level of glutathione was observed in roots, thus suggesting the rapid consumption of glutathione for Cd-chelation (Lee et al., 2010). GSTs are enzymes involved in the detoxification of cells in plants by conjugating glutathione with a wide range of molecules such as Cd. Two GSTs (OsGSTF14 and OsGSTU6) were overexpressed in rice roots after exposure to Cd (Cao et al., 2017). The S-glutathionylated conjugate metabolites were compartmentalized in plant vacuoles, preventing

root-to-shoot translocation, and reducing Cd-accumulation in the above-ground organs (Cao et al., 2017). In leaves, proteins involved in the synthesis of metabolic energy and redox power for Cd-detoxification were induced upon short-term Cd-stress, especially the vacuolar proton-ATPase (also elevated in roots) (Lee et al., 2010). The vacuolar proton-ATPase plays an important role in Cd transport into the vacuoles as free ions or as conjugates with glutathione or PCs. Thus, vacuolar sequestration is an important Cd-detoxification mechanism in rice leaves, as observed in Cd-hyperaccumulators (Isaure et al., 2015).

Finally, it is imperative to mention that a rice quantitative trait locus (QTL) encoding a defensin-like protein was identified, and termed as Cd accumulation in leaf 1 [CAL1] (Luo et al., 2018). Defensins are a group of small, cysteine-rich proteins, involved in innate immune responses and antifungal activity in various organisms (Holly et al., 2017). CAL1 was preferentially expressed in rice root exodermis and xylem parenchyma cells. CAL1 acts by chelating Cd in the cytosol and facilitating its extrusion to extracellular spaces, thus lowering cytosolic Cd levels while also enhancing long-distance Cd transport through xylem vessels (Luo et al., 2018). Significantly, CAL1 did not affect the accumulation of Cd and essential metals in grains, hence providing researchers with an option of breeding dual-function rice varieties that produce safe, non-toxic grains while also remediating soils.

2.5. Multifarious Cd tolerant or hyperaccumulating plants

Crassulaceae species are considered to be Cd-hyperaccumulators, which can accumulate excessively high levels of Cd in the aerial parts (mainly leaves) without any apparent toxicity (Fig. 1). Of them, *S. alfredii* and *S. plumbizincicola* can accumulate Cd up to 11,000 and 7000 µg Cd g⁻¹ DW, respectively (Liu et al., 2017; Zhang et al., 2017). Thus, they are promising candidates for the phytoremediation of Cd-contaminated soils.

To elucidate the underlying molecular mechanism of Cd-hyperaccumulation in *S. alfredii* and *S. plumbizincicola*, protein profile changes in leaf, root, and stem were analyzed using gel-free proteomic approaches (Zhang et al., 2017; Zhu et al., 2021, 2022). Through a comparison of the hyperaccumulating population (HP) with the non-hyperaccumulating population (NHP) of *S. alfredii* under Cd stress, several Cd-inducible DAPs were identified, of which the elevated DAPs in the "HP" included Matrix metalloproteinase-3, Heavy Metal ATPase-3 (HMA3), nonspecific lipid-transfer proteins (nsLTPs), SOUL heme-binding proteins, pollen allergen-like proteins, and short-chain dehydrogenase/reductase. These DAPs were mainly involved in heavy metal translocation, cell wall/membrane modification, carbohydrate and energy metabolism, plant defense, and stress responses (Zhang et al., 2017). A recent study involving proteomic analysis, identified a total of 352 Cd-induced transport proteins in *S. plumbizincicola*, including ABC transporters, ion transporters, aquaporins, proton pumps, and organic transporters; and the levels of these transport proteins enhanced significantly with the increasing length of exposure to Cd in the root (mostly), stem, and leaf (Zhu et al., 2022). Heterologous expression of SpABC28, SpMTP5, SpNRAMP5, and SpHMA2 in yeast and subcellular localization demonstrated Cd-specific transport activity (Zhu et al., 2022). Even though several transport-related proteins have been identified in Cd-hyperaccumulating plants through proteomic analyses, their specific functions and interactions in Cd-hyperaccumulation physiology need to be evaluated.

Phytolacca americana is a perennial plant frequently found growing in mining areas in soil contaminated with heavy metals (Fig. 1). Being a Cd-hyperaccumulator, *P. americana* can accumulate more than 240 µg Cd g⁻¹ DW in leaves (Peng et al., 2008) and up to 100 µM in shoots (Liu et al., 2010). The traits essential for Cd-hyperaccumulating ability like fast growth rates and high biomass identify *P. americana* as a potentially important species for phytoremediation of Cd-contaminated soils. After 48 h of exposure to 400 µM Cd, 25 DAPs were identified in *P. americana* leaves, which were mainly involved in

sulfur metabolism, transcription/translation, antioxidant systems, and redox reactions. The key enzymes involved in the methionine and methyl cycle were particularly upregulated under high-Cd-stress, whereas those involved in the Calvin cycle were downregulated (Zhao et al., 2011), indicating that the photosynthetic pathway was susceptible to Cd-induced stress even in hyperaccumulator plants.

Amaranthus gangeticus (Amaranth) is a leafy vegetable commonly consumed in East Asia (Fig. 1). It is recognized as a Cd-hyperaccumulator (Zhou et al., 2013). Comparative proteomic studies in two amaranth cultivars contrasting in Cd-accumulation capability indicated that the proteins involved in the glutathione-related pathway were accumulated in high levels in the high-Cd-accumulation cultivar 'Pen', resulting in activation of the translocation of Cd from root to shoot; whereas in the low-Cd-accumulation cultivar 'Nan', the retention of Cd in the roots was enhanced resulting in a reduction in the amount of Cd being transported into the shoot (He et al., 2018). 'Pen' exhibited stronger Cd-tolerance than 'Nan' due to the elevated DAPs involved in the detoxification of Cd. Thus, these results suggest a genotype-dependent Cd-tolerance and accumulation ability in amaranth.

Linum usitatissimum (Flax) is an herbaceous annual plant, cultivated for its fiber and seeds (Fig. 1). It accumulates large quantities of heavy metals (e.g., Cd) due to its well-developed root system and large biomass (Rehman et al., 2021). The cv. 'Jitka' showed a higher tolerance to elevated Cd levels in the soil than the cv. 'Tábor.' Comparative proteome-based analysis in the suspension cells of these two cultivars identified 14 Cd-induced DAPs, which were related to disease/defense, metabolism, protein transport and storage, signal transduction, energy, and cell structure (Hradilová et al., 2010). Ferritin and GST were upregulated under Cd-stress in 'Jitka' alone implying that the binding of ferritin and small thiol peptides with Cd contributed to Cd-tolerance in 'Jitka' due to the maintenance of lower levels of free Cd in cells. The other Cd-induced DAPs included fiber annexin, isoflavone reductase, S-adenosyl-L-methionine synthetase, and methionine synthase. These results were consistent with the Cd-detoxification mechanisms involving chelation, operated by both small thiol peptides (glutathione and PCs) and heavy metal-binding proteins (ferritin and lipocalin). Finally, the genes of the DAPs identified may facilitate the production of transgenic flax lines with enhanced Cd-tolerance and accumulation capacities for remediating Cd-contaminated soils.

Nicotiana tabacum (Tobacco) is a model dicot plant and an important cash crop globally. It can accumulate large amounts of heavy metals in its large-sized leaves (Fig. 1). Proteomic changes in the leaves of two cultivars with contrasting Cd-tolerance traits 'Yunyan2' (tolerant) and 'Guiyan1' (sensitive) were compared using hydroponic experiments with 50 μM Cd (Xie et al., 2014). A total of 18 Cd-induced DAPs were identified, of which epoxide hydrolase, enoyl-acyl-carrier-protein reductase, NPALDP1, chlorophyll a/b binding protein-25, HSP-70, and 14-3-3 proteins accumulated more copiously in 'Yunyan2' than in 'Guiyan1'. The elevated levels of photosynthesis-related proteins like NPALDP1, CAB25, GAPDH subunit B, and OEC3 may effectively protect the photosynthetic apparatus against Cd-induced toxicity (Xie et al., 2014). Similarly, thioredoxin-like proteins may stabilize the electron transfer chain, thereby promoting ATP synthase activity and alleviating Cd-induced photoinhibition of PSI and PSII; and maintaining the rates of chlorophyll synthesis in tobacco leaves under Cd-stress (Zhang et al., 2020a, 2020b).

Solanum lycopersicum (tomato) cultivars, 'Pusa Ruby' (PR, Cd-tolerant), and 'Calabash Rouge' (CR, Cd-sensitive) demonstrated contrasting properties in Cd-tolerance and accumulation. Proteomic analysis revealed that the significantly enhanced DAPs in 'CR' roots were mostly intracellular proteins, whereas those in 'PR' roots were extracellular and envelope proteins (Rodríguez-Celma et al., 2010). Moreover, the accumulation of DAPs in roots was Cd-dose-dependent: those involved in the glycolytic pathway, TCA cycle, and respiration were accumulated under low-Cd (10 μM) stress, whereas those involved in

Cd-detoxification (GST) were accumulated under high-Cd (100 μM) stress. Recently, a large number of Cd-responsive phosphoproteins were identified in leaves of 'PR' and 'CR'; and the levels in the abundance of ABC transporters, chaperones, HSPs, phosphatases, kinases, kinase/phosphatases, phytohormone-related proteins, and transcription factors differed in 'PR' and 'CR' under Cd-stress (Table 1, Marques et al., 2021). These results indicated an underlying proteomic basis for the differential and contrasting Cd-tolerance traits in both tomato cultivars.

Helianthus annuus (Sunflower) is considered to be a hyperaccumulator due to its ability for high-Cd-accumulation in shoots (Fig. 1) (Lopes Júnior et al., 2015). It is tolerant to long-term Cd-exposure (50 or 700 mg Cd per 770-mL-pot for 45 days). Proteomic analysis revealed that photosynthesis was the main metabolic process affected by high Cd concentrations, with the DAPs identified in leaves being involved mainly in energy synthesis and stress response, like ribulose-1, 5-bisphosphate carboxylase (RuBisCO), transketolase, and HSPs (Lopes Júnior et al., 2015).

Some woody species such as poplar are efficient in the uptake of heavy metals, mainly due to their highly developed root system (Fig. 1) (Zacchini et al., 2009), and a high capacity for storing metals in the trunk permanently (Harada et al., 2010). Many clones of *Populus* exhibit a high-Cd-accumulation potential with no apparent physiological toxicity (Unterbrunner et al., 2007). Hence, *Populus* trees have widely been used as a model woody species in phytoremediation of Cd-contaminated soils (Laureysens et al., 2005; Utmazian et al., 2007; Marmioli et al., 2013; De Oliveira and Tibbett, 2018; El-Mahrouk et al., 2020). A comparative proteomic study of *Populus* leaf and cambial tissue revealed an important tissue-specific response under Cd-stress (Durand et al., 2010). To screen the poplar clones for suitability to phytoremediation, the proteomic changes in the leaves of three clones were compared under Cd-stress (Marmioli et al., 2013). The results obtained demonstrated that proteins related to defense, stress response, and carbohydrate metabolism were significantly influenced as was observed in previous studies (Kieffer et al., 2008, 2009a, 2009b; Durand et al., 2010). The proteins associated with the maintenance of photosynthetic capacity and amino acid synthesis were found to be abundant in the hybrid clone A4A, indicating that A4A was more tolerant to short-term Cd-stress when compared to the other two clones. However, the effects of long-term Cd-stress on A4A growth rate and biomass production need to be ascertained before the selection of A4A for large-scale application in the phytoremediation of Cd-polluted soils.

3. Insights into Cd-tolerance, translocation, and accumulation in plants based on proteomic studies

Cd-hyperaccumulation in plants requires enhanced root uptake, increased xylem loading, augmented translocation from roots to shoots, and then final sequestration into vacuoles and cell walls in shoots (Uraguchi et al., 2009; Verbruggen et al., 2009; Isaure et al., 2015). In this section, we discuss the vital aspects of Cd-relevant processes in plants and highlight the differences in Cd-tolerance and accumulation traits across plant species based on physiological, biochemical, and proteomic data (Table 2).

3.1. Cd response and tolerance

Being a non-essential element, Cd is highly toxic to plants even at very low concentrations. At the cellular level, Cd causes oxidative stress, disrupts membrane integrity via excessive production of ROS, and interferes with the functioning of various cellular processes (Cuyppers et al., 2010). The aerial parts of plants exhibit conspicuous symptoms under prolonged Cd-stress, e.g., chlorosis, retarded growth, senescence, and substantial loss in crop yield (Cuyppers et al., 2010; Zhang et al., 2021). Even the physiology and morphology of Cd-tolerant plants, such as water hyacinth, are adversely impacted by Cd-stress (Wang et al., 2012). The effects of Cd-toxicity are more prominent in algae or hydrophytes

Table 2
The increased DAPs and pathways in Cd hyperaccumulators and/or Cd-tolerant plant materials under Cd stress.

Commonly increased DAPs under Cd stress	Increased pathways and their roles in response to Cd	Plant species	Reference
Proteins involved in starch degradation, gluconeogenesis, and N/S assimilation, antioxidant enzymes and HSPs	Energy supply, Cd chelation and detoxification	<i>Chlorella sorokiniana</i>	León-Vaz et al. (2021)
Enzymes of glycolysis and the TCA cycle, e.g. pyruvate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and enolase	Glycolysis, TCA cycle in leaf, providing metabolic energy and redox power for Cd detoxification	Poplar, rice, soybean, tomato, <i>Sargassum fusiforme</i>	Kieffer et al. (2009a); Lee et al. (2010); Rodríguez-Celma et al. (2010); Hossain et al. (2012); Yang et al. (2015a,b); Zhang et al. (2015)
Antioxidants and ROS scavenging enzymes, e.g. glutathione, thioredoxin, peroxiredoxin, peroxidase, ascorbate oxidase, NADH dehydrogenase, superoxide dismutase, phospholipase D	Anti-oxidative system, Cd tolerance, detoxification and oxidative stress defense	<i>Chlamydomonas reinhardtii</i> , soybean, rice, cucumber, poplar	Gillet et al. (2006); Sobkowiak and Deckert (2006); Ahsan et al. (2007); Cai et al. (2011); Hossain et al. (2012); Sun et al. (2016); Yang et al. (2016); Zhong et al. (2017); Huang et al. (2019)
Stress proteins, e.g. pathogenesis-related proteins	Stress defense	Poplar, rice	Kieffer et al., (2008, 2009b); Lee et al. (2010); Lomaglio et al. (2015); Yang et al. (2015a,b)
Glycolipid transfer protein	Membranes protection and stabilization.	Rice	Cai et al. (2011)
Chaperone, heat shock proteins, 14–3–3 protein, zinc finger protein	Protein synthesis, folding and transport, degradation	<i>Physcia adscendens</i> , poplar, tomato	Rustichelli et al. (2008); Huang et al. (2019); Marques et al. (2021)
Transcription factor, e.g. eukaryotic translation initiation factor (eIF)	Protein metabolism, protein synthesis, folding and transport, Cd signaling	Poplar, tomato	Huang et al. (2019); Marques et al. (2021)
Kinase, kinase/phosphatase	Glutathione metabolism	Tomato	Marques et al. (2021)
Glutathione S-transferase		Soybean, <i>Physcia adscendens</i> , rice	Sobkowiak and Deckert (2006); Rustichelli et al. (2008); Lee et al. (2010); Cao et al. (2017)
Histone H2B, chalcone synthase, proteins associated with lignin biosynthesis	DNA replication and repair, stress response, Cd-chelating pathways	Soybean, wheat	Sobkowiak and Deckert (2006); Ahsan et al. (2012); Jian et al. (2020)
Wall proteins, e.g. pectin methylesterase	Cd defence, cell wall restructuring and lignification in leaf and shoot	Poplar, alfalfa	Yang et al. (2015a,b); Gutsch et al. (2018a,b); Gutsch et al. (2019)
HMA, ABC transporters	Cd transport and localization	Cucumber, tomato	Gong et al. (2017); Marques et al. (2021)

with the whole plant being exposed to Cd present in the water. The cell structure of microalgae is especially vulnerable to Cd-toxicity due to remarkable changes in thylakoid ultrastructure (Mota et al., 2015) and an increase in the number and size of vacuoles (León-Vaz et al., 2021).

Proteomic studies have shown that Cd significantly influenced general defense and stress responses, and various metabolic pathways especially photosynthesis in a Cd-dose and -duration-dependent manner (Table 2). For example, levels of most enzymes involved in carbon fixation and light-dependent reactions (e.g., RuBisCO, carbonic anhydrase) were significantly diminished in poplar leaves both under short- and long-term exposure to Cd (14–56 days) (Kieffer et al., 2008, 2009a; Durand et al., 2010; Visioli et al., 2010; Marmiroli et al., 2013; Yang et al., 2015b; Dai et al., 2020), whereas the levels of enzymes involved in respiration (e.g., glycolysis and TCA cycle) were significantly increased (Durand et al., 2010; Yang et al., 2015b). The enhanced respiration rates in leaves supply more energy to plants adapted to long-term Cd-stress. Obviously, Cd-induced changes in the proteome profile shared some similarities across species but often exhibited specificity across plants at the organ, tissue, and even cellular levels (Table 2). The data from proteomic studies suggested that the changes in protein profiles correlated well with the physiological, morphological, and structural changes observed in plants under Cd-stress. Most Cd-inducible DAPs were localized to the cell wall, plasma membrane, vacuole, and cytosol indicating that these organelles are not only primary sites for Cd-sequestration and stress response but are also highly susceptible to Cd-toxicity.

Proteomic analysis demonstrated that plants employ similar DAPs to alleviate Cd-stress across species (Yang et al., 2015b). For example, unicellular algae (Mota et al., 2015; León-Vaz et al., 2021) and soybean suspension cells (Sobkowiak and Deckert, 2006) demonstrated similar Cd-induced proteomic changes, such as elevated levels of stress-related proteins and ROS scavenging enzymes. Several types of proteins, involved in cellular functions such as photosynthesis, energy and

carbohydrate metabolism, transcription and translation, oxidation and reduction, and stress responses exhibited similar patterns of change across most plants studied (Table 2, Fig. 2).

To cope with Cd-stress, plants whether Cd-tolerant or Cd-sensitive, employ a range of cell detoxification mechanisms (Fig. 2). Apart from extrusion through the plasma membrane, the most common mechanisms to minimize the Cd²⁺ toxicity include chelation with thiol ligands (e.g., glutathione and PCs), sequestration into the vacuole (Verbruggen et al., 2009; Isaure et al., 2015), and immobilization into the cell wall (Wang et al., 2021a, 2021b). Cd-chelation with PCs, sequestration into vacuoles, and efflux into the apoplast were observed in both the hyperaccumulator *A. halleri* and the non-accumulator *Arabidopsis lyrata*, but at different levels of intensity (Isaure et al., 2015). Thus far, three types of transporters involved in sequestering of Cd into vacuoles have been identified: CAX-type antiporters (Korenkov et al., 2009), Heavy Metal ATPases (e.g., HMA3) (Morel et al., 2009; Ueno et al., 2010; Miyadate et al., 2011; Krämer, 2010; Isaure et al., 2015) and PCs transporters [ABCC1 and 2] (Mendoza-Cózatl et al., 2011; Park et al., 2012).

The extent of Cd-tolerance varied not only across plant species, populations, and crop varieties (e.g., Zhao et al., 2006; Ahsan et al., 2012; Li et al., 2021), but also at the level of organs, especially roots, and leaves. The impact of Cd-toxicity was more prominent in roots than in leaves (Fig. 2). This can be explained by the early induction of some stress-related proteins, such as HSPs and GST in roots, while most proteins involved in primary metabolism pathways like glycolysis, TCA cycle, and N/S metabolism were significantly depressed (Kieffer et al., 2009b). The potential for Cd-resistance in plants can be improved through genetic engineering techniques (Ueno et al., 2010), leading to increased efficiency in the species employed for the phytoremediation of Cd-contaminated soils.

3.2. Root-shoot translocation of Cd

In terrestrial plants, roots are the first organ to be exposed to Cd-toxicity. Cd^{2+} enters the roots through apoplastic binding and/or symplastic uptake. Apoplastic binding involves Cd^{2+} influx through the process of sorption. Cd^{2+} enters into the symplast via plasma membrane-bound transporters/channels dedicated to divalent cations (e.g., Zn^{2+} , Fe^{2+} , Ca^{2+} , and Mg^{2+}) but with low specificity for Cd^{2+} . After root uptake, Cd is transported via the apoplast or symplast to the endodermal boundary, then loaded into the xylem, and eventually transported across a long distance to the shoots (Fig. 3) (Clemens and Ma, 2016; Song et al., 2017). Efficient translocation of Cd from roots to shoots, especially a high rate of xylem loading is physiologically crucial for enhanced hyperaccumulation capability (Lu et al., 2008). Xylem loading of Cd^{2+} in the hyperaccumulator, *A. halleri* is a rapid, energy-dependent process, which appears to be associated in part with the Zn^{2+} and Fe^{2+} translocation pathways. Cd is predominantly translocated as aqueous free Cd^{2+} ions through the xylem vessels (Ueno et al., 2008).

Hyperaccumulators usually have a greater capacity for translocating Cd^{2+} from roots to aerial parts when compared to low-Cd-accumulating plant species. In rice, root-to-shoot Cd translocation through the xylem is the major process to influence the accumulation of Cd in shoots and grains (Uraguchi et al., 2009). The root-to-shoot Cd translocation requires the loading of symplastic Cd^{2+} into the xylem by Heavy Metal ATPases (Pence et al., 2000; Papoyan and Kochian, 2004; Hanikenne et al., 2008; Wong and Cobbett, 2009) or by other transporters such as CAL1 in rice (Luo et al., 2018). *HMA4* encodes a P-type ATPase that loads Zn^{2+} and Cd^{2+} into the xylem. High expression of *HMA4* was necessary for Zn- and Cd-tolerance and hyperaccumulation in *A. halleri* (Hanikenne et al., 2008; Willems et al., 2010). Root-selective expression of *AtCAX4* and *AtCAX2* resulted in reduced foliar Cd-accumulation in field-grown *N. tabacum* (Korenkov et al., 2009).

Thus far, studies on Cd^{2+} uptake and translocation in roots mainly involved proteomic analysis of whole roots. The identification of changes in the proteome profile at subcellular sites like cell walls and vacuoles, and transfer cells is the key to revealing the mechanisms underlying the uptake, translocation, and sequestration of Cd^{2+} in hyperaccumulators. Several Cd-inducible transport proteins in the roots of *S. plumbizincicola* were observed to be highly upregulated (Zhu et al., 2022). These transporters were located either in the plasma membrane

or in the tonoplast. The mechanism behind the coordination of these transporters with each other in Cd translocation and sequestration in hyperaccumulators remains unclear.

3.3. High-Cd-accumulation in shoots

The aerial parts of a plant, especially the leaves are major organs involved in sequestering Cd. Genome-wide association studies indicated *HMA3* to be the primary determinant of natural variation in the Cd-accumulation potential of *A. thaliana* leaves (Chao et al., 2012). As observed in roots, Cd-chelation with glutathione and PCs in leaves is an important process in minimizing Cd-toxicity both in Cd-tolerant plants (Clemens, 2001; Park et al., 2012) and in hyperaccumulators (Verbruggen et al., 2009). Cd mainly accumulates in the xylem, phloem, and mesophyll tissue in leaves, with a Cd-reallocation process (Isaure et al., 2015). Mesophyll is a major storage site considering its volume. At the cellular level, vacuoles and cell walls were identified to be the major sites for Cd sequestration and accumulation in leaves (Fig. 4).

Owing to its large volume, the vacuole is the most important final storehouse for toxic heavy metals like Cd^{2+} . Vacuolar sequestration can protect the cytoplasm against metal toxicity (Isaure et al., 2015). Many metal transporters were identified in purified tonoplasts from barley leaves exposed to Cd-stress through proteome-based analysis, including CAX1 (induced by low Cd^{2+}) and AtMRP3, an ABC transporter homolog (induced by high Cd^{2+}) (Schneider et al., 2009). CAX1a might play a role in vacuolar Cd^{2+} transport. AtMRP3 was able to partially rescue a Cd^{2+} -sensitive yeast mutant. In addition, high expression of *HMA3* allowed storage of Cd in the vacuoles of *T. caerulescens* by functional characterization of *HMA3* (Ueno et al., 2011), the distribution of Cd in different tissues of *S. alfredii* was detected by using X-ray micro-fluorescence assay (Tian et al., 2011). The roles of these transporters in Cd^{2+} detoxification should be further investigated.

Cell walls are another important storage point of Cd in the aerial parts of plants (Fig. 4). For instance, up to 35 % of Cd was localized in the cell walls of mesophyll and epidermal cells in the Cd-hyperaccumulator, *T. caerulescens* (Cosio et al., 2005). In leaves of *M. sativa* plants exposed to Cd, most DAPs with increased abundance (163/212) from cell wall fractions were identified as secretory proteins involved in defense and cell wall restructuring, especially pectin methyl-esterase (Gutsch et al., 2018a, 2018b). These DAPs in cell walls were

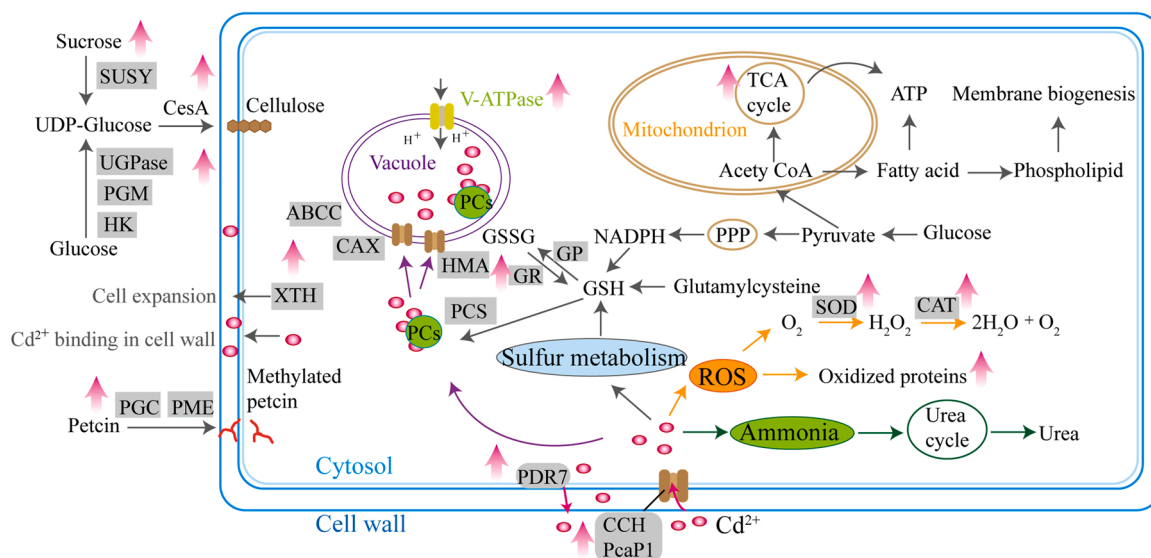


Fig. 2. The key proteins and pathways involved in Cd response and accumulation in plant roots. Thick red arrows indicate the Cd-inducible increased proteins. CAX, cation/ H^+ exchanger; CCH, copper transport protein; GP, glutathione peroxidase; GR, glutathione reductases; HMA, heavy metal ATPases; HK, hexokinase; PcaP1, plasma membrane-associated cation-binding protein 1; PCS, phytochelatins; PDR7, pleiotropic drug resistance 7; PGC, polygalacturonase; PGM, phosphoglucosyltransferase; PME, pectin methyl-esterase; SUSY, sucrose synthase; UGPase, UDP-glucose pyrophosphorylase; XTH, xyloglucan endotransglucosylase/hydrolase.

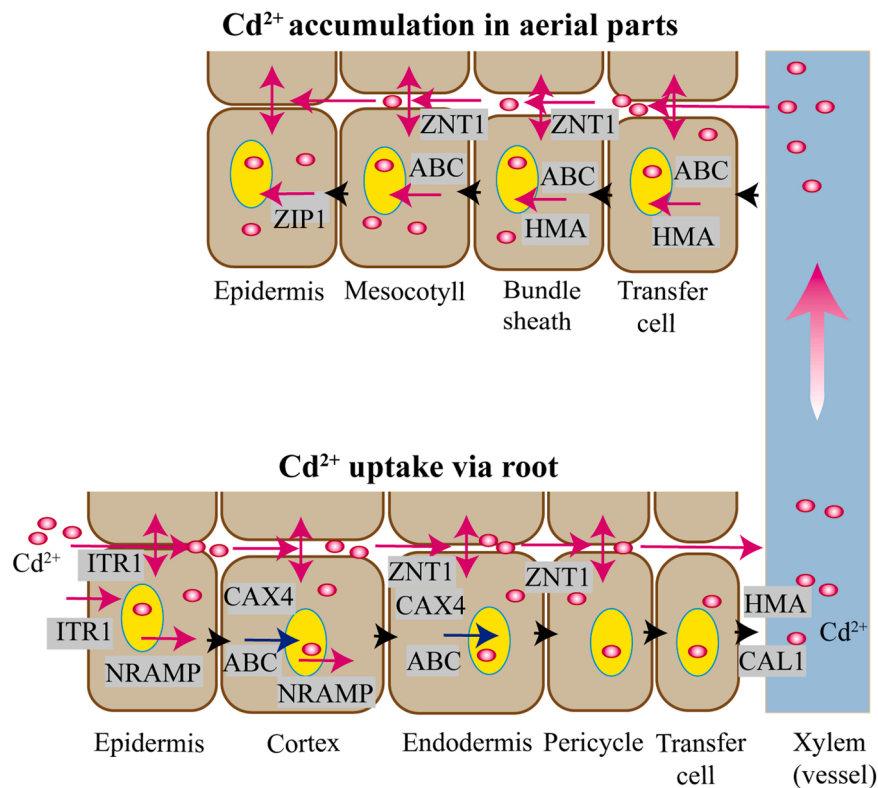


Fig. 3. Cd translocation from plants roots to aerial parts by xylem. The key major metal transporters involved in Cd transport and accumulation in roots and aerial parts were highlighted. The vacuoles are displayed in yellow. ZNT1, Zinc transporter 1; ITR1, myo-inositol transporter 1; NRAMP, natural resistance-associated macrophage protein; CAX4, cation/H⁺ exchanger 4; CAL1, Cd accumulation in leaf 1; ABC, ATP-binding cassette transporter; HMA, heavy metal ATPases.

mainly involved in defense response, cell wall lignification, and carbohydrate metabolism corroborating previous observations that the cell wall acts as an effective barrier against Cd-stress. Proteomic analyses additionally revealed that a majority of the DAPs induced by Cd-stress in *Populus* leaves were localized in chloroplasts and mitochondria, suggesting important roles of these organelles in the response and adaptation of plants to Cd-stress (Visioli et al., 2010).

However, the functions of a large number of Cd-induced proteins in leaves are not yet identified. Emphasis needs to be placed on studying the importance of the DAPs elevated on exposure to Cd, as they may be essential in recalibrating metabolism under stress conditions (Gong et al., 2015). The DAPs commonly induced by Cd-stress in a variety of plant species and the metabolic pathways with which they are associated are summarized in Table 2. These DAPs mainly include stress-related proteins (e.g., HSPs), enzymes of glycolysis and the TCA cycle, antioxidants, ROS scavenging enzymes, transcription factors, kinases/phosphatases, GST, cell wall proteins, and Cd²⁺ transporters among others. These upregulated, Cd-inducible DAPs would be more important for Cd-adaptation in plants than the depressed DAPs, an important point to be remembered when developing Cd-tolerant plants through breeding or genetic engineering. High levels of accumulation of Cd in the harvestable, aerial parts of plants provide insights into a new prospect for efficient phytoremediation of Cd-contaminated environments.

4. Methodological deficiencies in proteomic analysis of Cd-stress in plants

Proteomic approaches have been widely used to explore the mechanisms underlying differential Cd-tolerance and accumulation across plant species, but some methodological deficiencies in these studies need to be addressed in the future.

Firstly, the proteomic experiments were not carefully designed. Cd-

induced proteomic changes in plants were mostly analyzed in hydroponic experiments with plants or their cells exposed to short-term Cd-toxicity (Table 1). The hydroponic experiments could not accurately mimic the real-life environment. The physical and chemical properties of the soil significantly affect Cd mobility in soil and Cd concentration in plant tissues (Albert et al., 2021). It is generally accepted that the expression of stress tolerance traits in plants is largely dependent on the interaction of genotype × environment (abiotic or biotic) (Wu et al., 2017). Plants often exhibit varying levels of Cd-tolerance at different stages of development (Gutsch et al., 2018a). Only a limited number of studies have been conducted by growing plants in Cd-contaminated soils while studying the effects of Cd-stress over the whole plant growth period (e.g., Gutsch et al., 2018a, 2018b). Therefore, it is necessary to enhance the number of proteomic studies in plants (especially crops) grown in Cd-contaminated fields or soils. The data obtained from such studies would help in screening varieties of existing crops for high-Cd-tolerance and accumulation traits for usage in large-scale remediation of Cd-contaminated soils.

Secondly, the methodology used, especially in protein extraction, separation, and identification is questionable. Extraction of high-quality proteins from plant tissues is very difficult, mainly due to interference from various secondary metabolites such as polyphenols, polysaccharides, and organic acids (Wu et al., 2014; Niu et al., 2018). Most proteomic studies utilized less-efficient aqueous extraction protocols, followed by 2D-gel-based separation, and Coomassie Blue visualization, often resulting in the identification of a limited number of Cd-induced DAPs by MS/MS (Table 1). In particular, the low abundance levels and hydrophobicity of membrane proteins make their extraction and resolution difficult, often failing in identifying proteins by using MS/MS (Kongpracha et al., 2022). To address these drawbacks, several methods such as ultracentrifugation, precipitation, and treatment with urea or alkaline solutions have been applied (Kongpracha et al., 2022). Only a few studies utilized sensitive or powerful techniques, such as 2D-DIGE

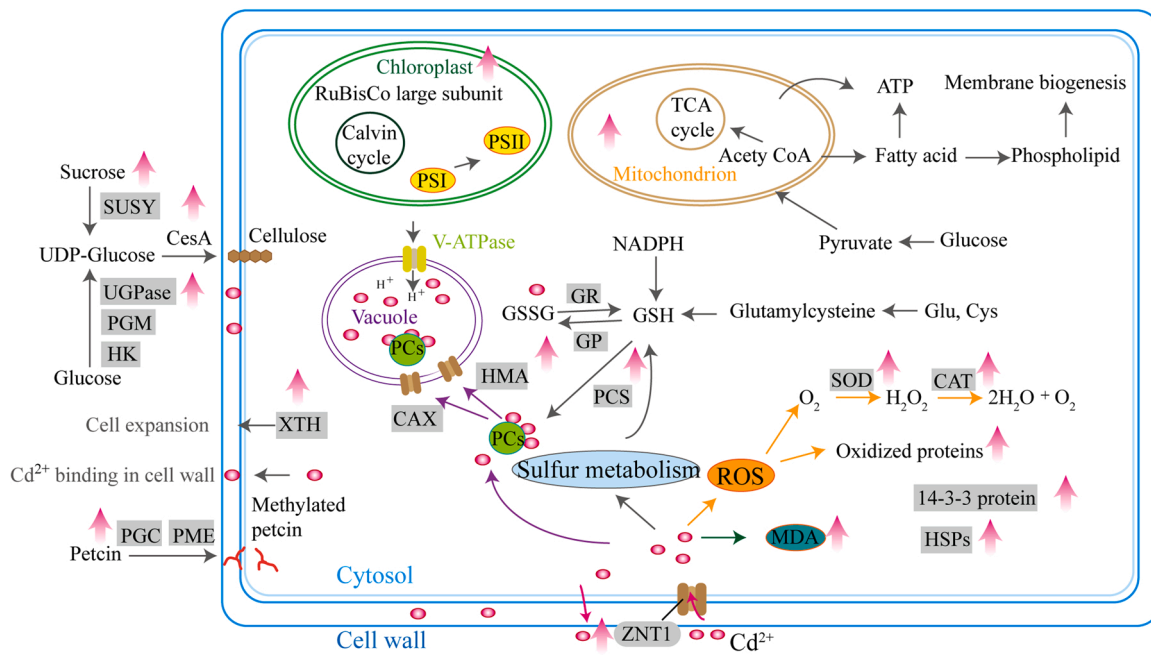


Fig. 4. The key proteins and pathways involved in Cd response and accumulation in leaves. Thick red arrows indicate the Cd-inducible increased proteins. The abbreviations are the same as in Fig. 2 legend.

(2D-Difference gel electrophoresis) (Gutsch et al., 2018a, 2018b), gel-free iTRAQ (isobaric tags for relative and absolute quantification), SILAC (stable isotope labeling by amino acids) (Schneider et al., 2009), and 2D-liquid chromatography (Visioli et al., 2010). Thus, the methods utilized need to be optimized for enhanced information on Cd-induced proteomic changes in plants.

Moreover, most studies were conducted at the whole plant or organ level, while only a handful of studies have investigated the subcellular (in isolated vacuoles and microsomes) and tissue-specific proteomic changes induced by Cd-stress (Schneider et al., 2009; Ahsan et al., 2012). Through the isolation of plasma membrane proteins in *S. plumbizincicola*, numerous novel transport proteins were identified, of which SpABC28, SpMTP5, SpNRAMP5, and SpHMA2 were validated for their Cd-specific transport activity in yeast (Zhu et al., 2022). Therefore, due to the cell specificity of proteomic responses (Gong et al., 2015), the protocols and techniques used in proteome-based analyses at the cellular or even subcellular levels need to be further updated, along with those employed at organ or tissue levels, to identify Cd-induced cell-specific responses in plants.

5. Conclusions and prospects for future research

This review focuses on proteomic studies in Cd-tolerant and -hyperaccumulating plants exposed to Cd-toxicity, including unicellular or multicellular algae, hydrophytes, model species, and common crops. Proteomic research revealed a set of Cd-induced DAPs that facilitates the understanding of the molecular mechanisms in hyperaccumulators involved in resistance to and accumulation of Cd or other heavy metals. However, identifying the specific functions of these DAPs is incomplete and the complex regulatory networks associated with them are largely unclear. Functional characterization of the Cd-responsive DAPs through knock-out or overexpression of their genes is necessary for engineering Cd-tolerant plants. Moreover, complex biological events usually involve the interplay of genes, transcripts, proteins, metabolites, and lipids. It is necessary to further strengthen the multi-omic analyses of Cd-induced-stress in Cd-hyperaccumulators and Cd-tolerant crops by involving integrative research.

Finally, phytoextraction efficiency depends largely on Cd-

accumulation capacity and plant biomass. Many plants face a conflict between higher-Cd-accumulation capacity and enhanced biomass production. It is imperative to develop Cd-tolerant plants for phytoremediation through genetic engineering and to breed Cd-resistant crop varieties, which allow higher-Cd-accumulation in straws but lower-Cd-concentration in grains for ensuring food safety.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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CRediT authorship contribution statement

LJN: Data curation, Writing and Editing the draft. CYL: Software, Visualization. JHZ, MS, WQ L, HL, FJT: Visualization, Reviewing and Editing the draft. XLH: Conceptualization, Funding acquisition. WW, XLW: Supervision, Conceptualization, Reviewing and Editing the draft. All authors read and approved the manuscript.

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.

Data Availability

No data was used for the research described in the article.

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Informed consent

Not applicable.

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