Chapter 3
Cadmium Accumulation by Plants of *Brassicaceae* Family and Its Connection with Their Primary and Secondary Metabolism

Petr Babula, Vojtech Adam, Ladislav Havel, and Rene Kizek

Abstract The mustard family – *Brassicaceae* – is well known as family of plants, metallophytes, which are able to accumulate wide range of heavy metals and metalloids, especially zinc and cadmium, but also nickel, thallium, chromium and selenium. Ecological importance of this process consists partially in plants themselves to survive negative environmental conditions. There are two basic different strategies, how to survive these conditions – accumulation of heavy metals in plants tissues with different intensity in individual cell types, but also organs, which is partially given by chemical composition of cell walls, and ability to synthesize special defensive – detoxification compounds rich on thiol groups – glutathione and phytochelatins, which are able to bind heavy metals and transport them to the "secure" cell compartment – vacuole. The second principle is based on ability to exclude heavy metals. Role of secondary metabolites rich on sulphur in detoxification of heavy metals is still discussed with unclear conclusions. Members of *Brassicaceae* family, especially genera *Thlaspi* and *Brassica*, are well-known hyperaccumulators of heavy metals with possible utilization in phytoremediation technologies. In this review chapter, mechanisms of cadmium uptake and transport and its deposition in various plant cells and tissues are discussed with respect with

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possible utilization in phytoremediation. In addition, role of special sulphur metabolites, which are typical for plants of *Brassicaceae* family – glucosinolates – in detoxification of heavy metals is discussed.

Keywords *Brassicaceae* • Cadmium • Glucosinolates • Metallophytes

Abbreviations

ABC	ATP-binding cassette transporter
APS	Adenosine 5′-phosphosulphate
ATP	Adenosine triphosphate
BSO	Buthionine sulphoximine
EDTA	Ethylenediaminetetraacetic acid
GCS	γ-Glutamyl cystein synthetase
γ-GC	γ-Glutamyl cystein
GSH	Glutathione reduced
GSSG	Glutathione oxidized
MT	Metallothionein
NTA	Nitrilotriacetic acid
OASTL	*O*-acetylserine(thiol)lyase
PC	Phytochelatins
PCS	Phytochelatin synthase
ROS	Reactive oxygen species

3.1 Family *Brassicaceae* – Its Characterization and Specificity

The mustard family – *Brassicaceae* (syn. *Cruciferae*) – is together with next 15 families classified in order *Brassicales*. Other important families are *Capparaceae*, which is sometimes included in *Brassicaceae* as subfamily *Capparoideae*, *Tropaeolaceae* and *Caricaceae*, which is well known for edible fruits – papaya – with interesting pharmacological properties (Breithaupt et al. 2003; Nayak et al. 2007; Okeniyi et al. 2007; Amazu et al. 2009; Abdullah et al. 2011). *Brassicaceae* (mustard family) consists of usually hermaphroditic herbs, sometimes shrubs, with simple lobbed or divided, spiral, exstipulate leaves (Fig. 3.1). Flowers are arranged in inflorescence represented usually by a raceme. Flowers are bisexual, usually actinomorphic, perianth is dichlamydeous, cruciate (cross-shaped). Androecium consists of six stamens (two outer are shorter than four inner), Gynoecium is syncarpous, consisting of two carpels. The fruit is a silique or silicle (Ehrendorfer and Neuffer 2006). Classification of *Brassicaceae* family (338 genera with 3,709 species) is very complicated, still under the discussion, see Table 3.1 (Crespo et al. 2000; Marhold et al. 2004; Johnston et al. 2005; Bremer et al. 2009; Haston et al.

Fig. 3.1 The most important members – hyperaccumulators – of the *Brassicaceae*. (1) *Arabidopsis halleri*, (2) *Arabidopsis thaliana*, (3) *Brassica juncea*, (4) *Thlaspi caerulescens*, (5) *Thlaspi praecox*

Table 3.1 Taxonomic classification of *Brassicaceae*

Phenetic classification[a]	Cladistic classification[b]
Kingdom: *Plantae*	Clade: *angiosperms*
Phyllum: *Magnoliophyta*	Clade: *eudicots*
Class: *Magnoliopsida*	Clade: *core eudicots*
Subclass: *Dilleniidae*	Clade: *rosids*
Superorder: *Capparaneae*	Clade: *malvids*
Order: *Capparales*	Order: *Brassicales*

[a]In accordance with Armen Takhtajan classification system
[b]APGIII classification (Bremer et al. 2009)

2009; Huang et al. 2010; Qiu et al. 2010; Al-Shehbaz et al. 2006; Bailey et al. 2006; Koch and Mummenhoff 2006; German et al. 2009; Endress 2011; Ge et al. 2011).

Family has a worldwide distribution. There are many species of economical value, for example vegetable plants, plants used for obtaining of dyes, oil, etc. There are also many ornamental plants. The best known member of family is *Arabidopsis thaliana*, plant noted as a model for molecular biology. The *Brassicaceae* contains high number of species that are able of hyperaccumulation of heavy metals, especially **nickel** (genera *Thlaspi* and *Alyssum*), **cadmium** and **zinc** (*Thlaspi caerulescens, Thlaspi praecox, Thlaspi goesingense* and *Arabidopsis halleri*). About 25% of all known hyperaccumulators are members of this family (Rascio and Navari-Izzo 2011; Marques et al. 2004; Doran and Nedelkoska 1999).

3.2 Cadmium as an Important Contaminant of Living Environment

Cadmium is soft, bluish-white metal that is chemically similar to zinc and mercury. It occurs as a minor component in zinc ores, no cadmium-containing ores are known. There is only one exception – greenockite (CdS) is closely associated with zinc ore sphalerite (ZnbS) (Criscuolo and Oconnor 1950; Schwartz 2000). Cadmium has many industrial applications. It is used in batteries, in electroplating, as a barrier to control nuclear fission, for manufacturing of pigments, as stabilizers of plastics, etc. Cadmium is in laboratories used for preparation of cadmium-selenide quantum dots, which emit luminescence after excitation (Beri et al. 2011; Fortes et al. 2011; Harris et al. 2011; Kumar and Biradar 2011). Cadmium has no biological role in the living organisms. However, marine diatoms (*Thalassiosira weissflogii*) contain cadmium-dependent enzyme carbonic anhydrase CDCA1, enzyme that catalyses the reversible hydration of carbon dioxide (Cullen et al. 1999; Lane and Morel 2000; Park et al. 2007, 2008). Cadmium performs the same function as zinc in this enzyme. It seems that cadmium is involved in function of this enzyme instead zinc ions, which may be replaced by cadmium ions (Xu et al. 2007; Strmecki et al. 2010). Cadmium is one of the most important contaminants of the environment. The steel industry and waste incineration followed by volcanic action and zinc production are estimated to account the largest emissions of atmospheric cadmium (Bleil and Albers 1964; Boutron et al. 1993; Milacic et al. 1995; Karar et al. 2006; Ostrowska 2008). The main source of soil and water cadmium contamination are especially fertilizers (phosphate fertilizers), but also pesticides, fungicides, and sewage (Mirlean and Roisenberg 2006; Chen et al. 2007; Zarkovic and Blagojevic 2007; Zhao and Masaihiko 2007; Yildiz et al. 2008; Hadlich and Ucha 2010; McGrath and Tunney 2010). Contamination of soil and water by cadmium is connected with its uptake by plants – crops and vegetables – cultivated for human consumption (Jafarnejadi et al. 2011; Khodaverdiloo et al. 2011; Moustakas et al. 2011; Romkens et al. 2011). Smoking represents one of the most important sources of humans' exposure to cadmium (Satarug and Moore 2004; Massadeh et al. 2010; Takeuchi et al. 2010; Lin et al. 2011). Numerous studies are focused on the formation of organic cadmium compounds by organisms, which are more toxic in comparison with inorganic forms, especially dimethyl derivatives (Yannai and Berdicevsky 1995). Toxicity of cadmium is known for many years. Chronic prolonged oral cadmium indigestion is known in Japan as Itai-Itai disease. Human toxic exposure causes damage of kidneys based on kidney tubular damage. Cadmium can also damage bones via direct effect on bone tissue and indirect effect associated with kidney dysfunction (Nishijo et al. 1996; Kasuya 2000; Ogawa et al. 2004; Inaba et al. 2005, 2006; Kobayashi et al. 2009). Just itai-itai disease is connected with damage of bones and is characterized by multiple fractures of the long bones in the skeleton (Wang et al. 1994). Carcinogenicity of cadmium is widely discussed (Barrett 2009; Chen et al. 2009; Strumylaite et al. 2009, 2010; Absalon and Slesak 2010; Gallagher et al. 2010; Julin et al. 2010; Talaat et al. 2010). It has been proposed as a prostate and

renal carcinogen, however, available data are still controversial (Kolonel 1976; Il'yasova and Schwartz 2005). Generation of reactive oxygen species, interactions with proteins, and induction of apoptosis by cadmium ions have been described in many *in vitro* studies. Toxicity of cadmium on plants includes inhibition of many enzymes involving in disruption of physiological processes including photosynthesis and growth alterations (Chaffei-Haouari et al. 2009; Hasan et al. 2009; Lopez-Millan et al. 2009; Rodriguez-Serrano et al. 2009; Cherif et al. 2011).

3.3   General Aspects of Cadmium Uptake, Translocation and Accumulation in the *Brassicaceae*

Family *Brassicaceae* contains some members, in which metabolism of heavy metals is intensely investigated. *Arabidopsis thaliana* represents the most important member of family and model plant in molecular biology. Cadmium is rapidly absorbed, translocated and accumulated in the aerial parts of many plants (Fig. 3.2). Tolerance of heavy metals is closely connected to regulation of sulphur uptake. Sulphate transporters are proteins involved in sulphate transport across a membrane. Two classes of sulphate transporters have been established – low -affinity and high-affinity. They differ not only in condition, under they work (sulphur-replete/ sulphur/deficient conditions) and in selectivity. Some of them are responsible for uptake of not only sulphate, but also chemically similar, but toxic analogues, such as selenate. High-affinity sulphate transporters are the best known and characterized sulphate transporters. They have been identified especially in model plant *Arabidopsis thaliana* with tissue specificity (Hawkesford 2000, 2003; Buchner et al. 2004; Maruyama-Nakashita et al. 2004; Kumar et al. 2011). Recent works describe newly discovered sulphate transporters in different plants. For example, low-affinity sulphate transporter BnSultr2;2 together with high-affinity sulphate transporter BnSultr1;1 has been identified in *Brassica napus*. Increased tolerance to cadmium ions based on the efficient sulphate uptake and assimilation has been identified (Sun et al. 2007). Some genes involved in cadmium uptake have been identified. BjCdR15 expression was detected mainly in the epidermis and vascular system of cadmium-treated *Brassica juncea* plants (Farinati et al. 2010). Further transport of cadmium (and next metal ions) into aerial parts is significantly affected by: (i) interactions with cell walls, (ii) chelation by thiols, (iii) compartmentation into vacuoles (Nocito: Cadmium retention). Transport of cadmium ions is via cells (across cell membranes) is closely connected with transporters (Fig. 3.2). In addition, a plenty of transporters associated with transport of next heavy metals (zinc, lead, arsenic, etc.) has been described (Song et al. 2010). Cadmium is usually compartmented in vacuoles. However, interaction and deposition in cell walls have been described (Basic et al. 2006). These findings are in agreement with next published data (Ni and Wei 2003). Cadmium accumulators *Arabidopsis halleri, Arabidopsis thaliana, Thlaspi caerulescens*, *Thlaspi praecox* and *Brassica juncea* are intensely studied due to their abilities to accumulate cadmium ions. Individual questions are discussed in independent chapters.
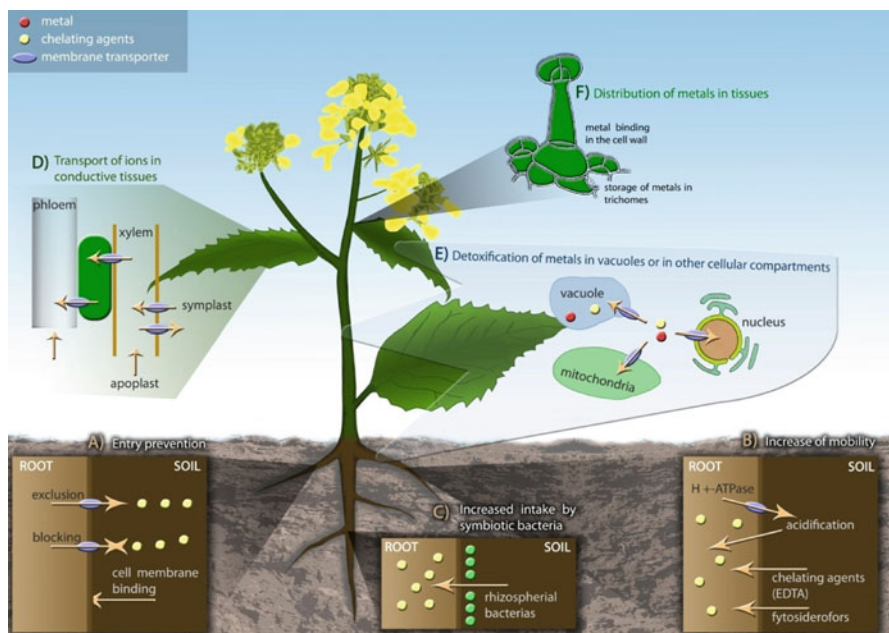
Fig. 3.2 Molecular mechanisms proposed to be involved in transition metal accumulation by plants. (**a**) Metal ions are mobilized by secretion of chelators and by acidification of the rhizosphere. (**b**) Uptake of hydrated metal ions or metal-chelate complexes is mediated by various uptake systems residing in the plasma membrane. Inside the cell, metals are chelated and excess metal is sequestered by transport into the vacuole. (**c**) From the roots, transition metals are transported to the shoot via the xylem. Presumably, the larger portion reaches the xylem via the root symplast. Apoplastic passage might occur at the root tip. Inside the xylem, metals are present as hydrated ions or as metal-chelate complexes. (**d**) After reaching the apoplast of the leaf, metals are differentially captured by different leaf cell types and move cell-to-cell through plasmodesmata. Storage appears to occur preferentially in trichomes. (**e**) Uptake into the leaf cells again is catalysed by various transporters [not depicted in (**f**)]. Intracellular distribution of essential transition metals (= trafficking) is mediated by specific metallochaperones and transporters localized in endomembranes (please note that these processes function in every cell). Abbreviations and symbols: *CW* cell wall, *M* metal, *filled circles* chelators, *filled ovals* transporters, *bean-shaped structures* metallochaperones (Modified according to Clemens et al. 2002)

3.4 Cadmium Uptake, Translocation and Deposition in *Arabidopsis thaliana*

Arabidopsis thaliana tolerates cadmium concentration of 1 μM in substrate (cultivation medium) without visible signs of the toxicity. However, higher concentrations (more than 5 μM) leads to the visible morphological changes including shortening the elongation zone of roots, premature xylogenesis, changes in rhizines formation and their premature elimination, and changes in formation of lateral roots. Changes in root system are closely connected with reduction of aerial parts growth as well as total biomass (Polec-Pawlak et al. 2005; Semane et al. 2007; Isaure et al. 2006; Van Belleghem et al. 2007). However, there are significant differences between individual studied members of the *Brassicaceae* as well as between

individual ecotypes. Especially reduction of biomass production under Cd supplementation represents one of the most important restriction of their usage in phytoremediation technologies (Kupper et al. 2000; Bert et al. 2002; Craciun et al. 2006; Zhao et al. 2006).

Cadmium exposure induces expression of about 80–100 genes, which encode especially proteinkinases, transcriptional factors, calmodulin-associated proteins, chaperones, and protective compounds, such as thiols and antioxidant enzymes, such as catalases, ascorbate peroxidases, superoxide dismutases, which are closely connected with cadmium detoxification and elimination of reactive oxygen species (Suzuki et al. 2001; Semane et al. 2007).

Arabidopsis thaliana is cadmium excluder. Roots of experimental plants can contain up to 89% of cadmium in dependence on experimental conditions. Only a minority of cadmium is transported into aerial parts – shoots (Polec-Pawlak et al. 2005; Isaure et al. 2006; Van Belleghem et al. 2007). Similar results have been obtained in related species – *Arabidopsis halleri*. In this species, roots represent the main organs of cadmium accumulation in hyperaccumulating ecotypes (Craciun et al. 2006). This fact is connected with the binding of cadmium ions into cell walls, especially in the lower concentrations (up to 1 μM). The negatively charged carboxyl group of the cell walls provides sites for cation exchange leading to the accumulation of positively charged divalent or polyvalent cations. Experimental conditions play crucial point in these experiments. Hydroponically cultivated *Thlaspi coerulescens* demonstrate the highest cadmium content in roots, however, in soil cultivated plants has only 20% of cadmium in roots, rest of cadmium ions is transported into aerial parts (Perronnet et al. 2003; Roosens et al. 2003). Precipitation of cadmium ions especially with phosphorus under formation of insoluble compounds has been demonstrated. Cadmium ions at concentration about 5 μM are transported into cortex, where form granular deposits in cell walls and intercellular spaces in *Arabidopsis thaliana*. Granular deposits in the cytoplasm are well evident at high concentrations (about 50 μM and more). Deposition of cadmium in cell walls and intercellular spaces is connected with disruption of water transport within roots. Casparian strips present in endodermis contain significantly higher amount of cadmium compared to cell walls of other cells. In addition, granular deposits as a result of cadmium treatment are well evident also in vacuoles. Radial transport of cadmium ions is connected with formation of granular deposits in pericycle and xylem. Higher cadmium content was detected in passage cells, especially in the form of cytoplasmic granular deposits.

In *A. thaliana*, cadmium is transported from roots in the complexes with phytochelatins. On the other hand, transport of cadmium in *A. thaliana* is bidirectional (Gong et al. 2003; Isaure et al. 2006). High content of cadmium in phloem, respectively in cytoplasm of sieve elements and companion cells, is a result of translocation of cadmium from aerial parts – shoots (Van Belleghem et al. 2007). Xylem sap of *A. hallerii* contains cadmium ions in the free form, or in the complexes with citrate (Ueno et al. 2008). In the aerial plant parts, the highest content of cadmium has been found in trichomes in *A. thaliana*. This fact is probably connected with the protection of photosynthetically active tissues – leave mesophyll – against toxic effect of cadmium ions. In comparison with trichomes, epidermis and leaf mesophyll contain undetectable cadmium amount.

Only minimal cadmium levels have been detected in vascular bundles of leaves, especially in vessel elements (Isaure et al. 2006; Van Belleghem et al. 2007). Different situation has been observed in *A. halleri*, whose populations in France may be considered as hyperaccumulators. The highest cadmium concentration was detected in the bases of trichomes (especially in the oxygen compounds as oxides or in the form of citrate) (Kupper et al. 2000). Nevertheless, treatment of experimental plants by high cadmium concentrations leads to its transport into leaf mesophyll vacuoles. Due to fact, that epidermal cells are only weakly vacuolated, they contain only minimal Cd concentrations (Kupper et al. 2000). Different mechanism of cadmium accumulation is presented in *T. coerulescens*, species lacking trichomes, which are usual place of this process. The highest cadmium content was detected in the big epidermal cells (Cosio et al. 2005; Leitenmaier and Kupper 2011) followed by leaf mesophyll (Ma et al. 2005). Thirty-five percent of cadmium ions is bound in cell walls of epidermal/mesophyll cells. The youngest leaves demonstrate the highest accumulation capabilities (Perronnet et al. 2003). Colenchyma cells, which serve as mechanical tissue, contain the highest cadmium content in *T. praecox* leaves (Vogel-Mikus et al. 2008).

Whereas cadmium in roots of experimental *A. thaliana* plants predominates in insoluble form (54% versus 46% of soluble form) due to formation of precipitates with phosphates under formation of $Cd_3(PO_4)_2$ (rhizodermis, apoplast of cortical cells), about 58% of total cadmium in shoots is in water-soluble form (Polec-Pawlak et al. 2005). Phosphate precipitates represent the main Cd form also in *A. halleri*. On the other hand, there are significant differences between individual members of the *Brassicaceae* (Ma et al. 2005). There are also differences between tissues of various onthogenetical ages (Perronnet et al. 2003). However, there is one fundamental question – isn't formation of these precipitates a result of hydroponic cultivation of experimental plants (Zhao et al. 2006)? Granular deposits in symplast are formed by cadmium coordinated to sulphur, which suppose reaction between cadmium ions and sulphur-containing molecules, especially Cd-phytochelatins, Cd-phytochelatins-sulphide and/or Cd-sulphide (Isaure et al. 2006; Van Belleghem et al. 2007). High-molecular ligands (cell walls with carboxyl groups) are responsible for cadmium depositions in shoots (Polec-Pawlak et al. 2005). 75% of total cadmium is in trichomes bound with O and N, probably in pectins and cuticle of cell wall, only 25% of cadmium is bound with sulphur, probably with GSH. This fact corresponds to increased levels of GSH in trichomes (Isaure et al. 2006).

3.5 Biochemical and Molecular Aspects of Cadmium in *Arabidopsis thaliana*

Exposition of *A. thaliana* to cadmium ions leads to the increased expression of the genes for *O*-acetylserine(thiol)lyase (OASTL), γ-glutamyl cysteine synthetase (GCS) and glutathione synthetase (GS). All these findings support importance of

sulphur compounds in cadmium detoxification (Lee et al. 2003). Expressions of GS and GCS are increased in the first step after cadmium exposition. Product of GCS-γ-glutamylcysteine (γ GC) – represents substrate for GSH biosynthesis. These connections have been verified using buthionine sulphoximine (BSO). Application of this inhibitor of GCS biosynthesis leads to the enhancement of sensitivity of *Arabidopsis thaliana* plants to cadmium ions (Wojcik and Tukiendorf 2011). Similar results were obtained using *cad2-1* mutant with altered genes for GCS, which produce only 30–45% of GCS in comparison with wild types (Cobbett et al. 1998). External application of GSH does not lead to the increased tolerance to cadmium, but it is toxic also for plants without Cd exposition. This fact indicates the role of GSH in biosynthesis of phytochelatins. Application of GSH may lead to the disruption of oxidation reduction balance in cells due to reduction of GSSG, which is formed from GSH in cultivation medium (Wojcik and Tukiendorf 2011). Role of phytochelatins (PCs) has been investigated using *Arabidopsis thaliana* *cad1-3* mutant with mutation in *AtPCS1* gene for phytochelatin synthase. Expression of *TaPCS1* from *Triticum aestivum* in roots of *cad1-3 A. thaliana* mutants led to the reduction of Cd accumulation in roots and enhancement of Cd transport from roots into shoots (Gong et al. 2003). Expression of *AtPCS1* is twofold increased after Cd application, but only in first 5 days (Lee and Korban 2002). Activity of AtPCS1 is after it regulated only on the enzymatic level. Presence of GSH-Cd is limiting factor for PC biosynthesis (Lee and Korban 2002; Lee et al. 2003). However, there are still unanswered questions in the regulation of PCs biosynthesis under Cd exposition (Semane et al. 2007). On the other hand, overexpression of *AtPCS1* does not increase production of PCs and leads to the increased sensitivity of experimental plants to Cd. This fact may be connected with disruption of metals homeostasis, toxicity of PCs excess or depletion of GSH (Lee et al. 2003). Enhanced expression of *AtMT2a* and *AtMT3* for metallothioneins like proteins (MTLs) after Cd exposition is connected with increased tolerance of plants to Cd. Expression of these genes in stomatal cells of *Vicia faba* increased their tolerance to Cd under reduction of ROS formation. MTLs are localised in cytoplasm, they are not translocated into vacuoles after Cd exposition in comparison with PCs. In addition, protective effect of MTLs is based on their antioxidant properties. This fact has been confirmed in experiments with hydrogen peroxide as a generator of ROS (Lee et al. 2004). Transporters of metals of the transport ATPase group – *AtHMA2*, *AtHMA3* and *AtHMA4* plays important role in complexation of heavy metals in cells. Heterological expression of *AtHMA3* in *ycf1* yeast mutants has confirmed role of this transporter in vacuolar sequestration of cadmium ions. Differences in the expression of *AtHMA2*, *AtHMA3* and *AtHMA4* are indicative to their different roles (Gravot et al. 2004). AtHMA2 and AtHMA4 are localised in plasmalemma, whereas AtHMA3 in tonoplast, so, it is well evident that AtHMA3 is involved in sequestration of toxic heavy metal ions into vacuoles. Highest *AtHMA2, AtHMA4* and *AtHMA3* expression was detected in the cells of root stele and in root vascular tissue as well as in stomatal cells and cells of hydatodes. All these cells are in the first contact with heavy metals ions – roots with soil/water and stomata and hydatodes with air polluted by heavy metals.

In addition, these tissues are essential for maintenance of physiological functions, so, detoxification of heavy metals in these tissues is fundamental. AtHMA2 and AtHMA4, which main functions consist in transport of zinc ions to the xylem, have significant affinity to cadmium ions and are able to execute their translocation from roots to shoots. These fact have been confirmed using mutants *hma4* (increased amount of Cd in roots, reduced transport to the shoots), and *hma2 hma4* (Cd accumulation is reduced to only 3% compared to wild type, 2–3-fold higher sensitivity to cadmium ions). HMA2 and HMA4 represent the main mechanism of Cd transport to the xylem. However, in comparison of *hma2 hma4* and *cad1* mutants, sensitivity of *cad1* mutant to cadmium ions is 10–20-fold higher (Wong and Cobbett 2009).

AtNRAMP1, AtNRAMP3 and AtNRAMP4 are the next transporters associated with cadmium transport. *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* are expressed in both roots and shoots. Expression of *AtNRAMP3* and *AtNRAMP4* is induced by iron deficiency; it means that their primary function consists in Fe transport. *AtNRAMP3* knock-out does not lead to the reduction of iron absorption (as a result of IRT1 transporter redundancy), but to the increase of tolerance of mutants to cadmium ions. Its overexpression is connected with increased sensitivity of plants to the Cd. In conclusion, AtNRAMP3 is able to transport both Fe and Cd ions, which means that its function consists in iron absorption under presence of Cd, which inhibits other Fe transporters (Thomine et al. 2000). *A. thaliana* genome contains ten *AtPCR* (plant cadmium resistance) genes, which are connected with cadmium resistance. *AtPCR1* and *AtPCR2* expression is induced by Cd exposition. Their expression is not connected with GSH. This fact has been verified by BSO application (Song et al. 2004).

PDR transporters play important role in host-pathogen interactions, enhanced expression of some *PDR* has been recorded under different stress conditions. *AtPDR8* is up-regulated at Cd and Pb exposition. Knockout or silencing of *AtPDR8* increases sensitivity of experimental plants to Cd, on the other hand, its overexpression reduces amount of accumulated Cd in plants and reduce rate of Cd absorption by individual protoplasts. AtPDR8 serves as a pump for transport of Cd ions out of cells (Kim et al. 2007). Mutation of gene *CDR3* – *cdr3-1D* – leads to the enhancement of tolerance of mutants to Cd, Pb, Cu, H_2O_2, and to the reduction of Cd and Pb accumulation. *Cdr3-1D* mutant has increased expression of *AtPDR8/AtPDR12*, *AtPCR1* and *GSH1* and increased levels of GSH. *CDR3* is responsible for regulation of above-mentioned genes (Wang et al. 2011). All transporters found in *A. thaliana* are summarized in Table 3.2. In addition, their comparison with next *Brassicaceae* species is presented.

Nitric oxide (NO), important signal molecule, plays crucial role in Cd metabolism in plants. Exposition of plants to cadmium ions leads to the enhancement of NO production in both roots and leaves. On the other hand, supplementation of plants by NO scavengers cPITO and/or L-NAME causes reduction of Cd accumulation in roots under reduction of inhibition of root growth. Nevertheless, cadmium accumulation in aerial parts – shoots – is not altered. All these facts mean that NO is responsible for inhibition of root growth. In addition, NO modulates functions of

Table 3.2 Cadmium transporters connected with its accumulation in the *Brassicaceae*

Transporters	Metals	Plants	Reference(s)
HMA3	Cd, Co, Pb, Zn	*A. thaliana* (AtHMA3) *Thlaspi caerulescens* (TcHMA3)	Gravot et al. (2004), Morel et al. (2009), and Ueno et al. (2011)
HMA2	Cd, Zn	*A. thaliana* (AtHMA2)	Eren and Arguello (2004), Eren et al. (2006, 2007), and Wong and Cobbett (2009)
HMA4	Cd, Cu, Pb, Zn	*A. thaliana* (AtHMA4), *A. halleri* (AhHMA4), *T. caerulescens* (TcHMA4)	Courbot et al. (2007), Hanikenne et al. (2008), and Grispen et al. (2011)
NRAMP1	Cd, Fe, Mn	*A. thaliana*	Curie et al. (2000) and Cailliatte et al. (2010)
NRAMP3	Cd, Fe, Mn	*A. thaliana* (AtNRAMP3) *T. caerulescens* (TcNRAMP3)	Wei et al. (2009) and Oomen et al. (2009)
NRAMP4	Cd, Fe, Mn, Zn	*A. thaliana* (AtNRAMP4) *T. caerulescens* (TcNRAMP4)	Lanquar et al. (2004, 2005, 2010), and Oomen et al. (2009)
NRAMP	Cd, Zn	*B. juncea* (BjNRAMP)	Das et al. (2011)
YSL	Cd, Zn	*B. juncea* (BjYSL)	Das et al. (2011)
PDR8	Cd	*A. thaliana* (AtPDR8)	Kobae et al. (2006) and Kim et al. (2007)
MRP6	Cd	*A. thaliana* (AtMRP6)	Gaillard et al. (2008)
MRP7	Cd	*A. thaliana* (AtMRP7)	Wojas et al. (2009)
IRT1	Cd, Fe	*A. halleri, T. caerulescens*	Zhao et al. (2006), Plaza et al. (2007), and Ueno et al. (2008)
ZNT6	Cd	*T. caerulescens*	Wu et al. (2009)
ZIP9	Cd, Fe, Mn, Zn	*T. caerulescens*	De Mortel et al. (2008)

calcium channels and pumps, because application of NO scavengers does not lead to the reduction of calcium content in roots after cadmium exposition in comparison with NO-scavengers untreated plants (Besson-Bard et al. 2009).

3.6 Connection Between Primary and Secondary Metabolism and Cadmium Hyperaccumulation

Sulphur belongs to the group of the most important essential nutrients in organisms. In plants, sulphur is taken up especially in the form of inorganic sulphate. After reduction, sulphur is incorporated into cysteine in the processes of primary sulphate assimilation. Cysteine serves as a sulphur donor for biosynthesis of both methionine, the major sulphur containing amino acid in plants and precursor of glucosinolates biosynthesis (Fig. 3.3), and glutathione, the most important protective non-protein thiol compound (Mugford et al. 2011). Glucosinolates, mustard oil glycosides, represent one of the bioactive sulphur-containing secondary metabolites in plants, which occur almost only in members of order *Brassicales*
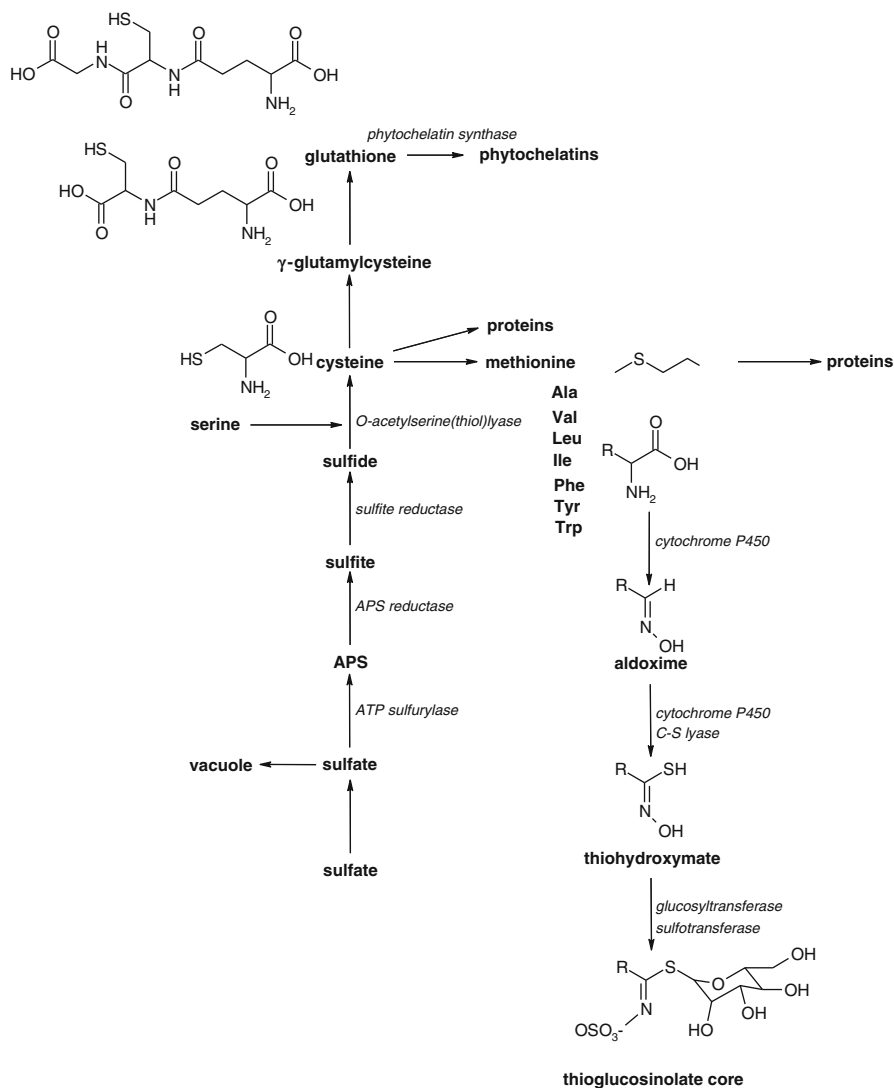
Fig. 3.3 Biosynthesis of thiols and glucosinolates. *APS* adenosine 5′-phosphosulfate, *ATP* adenosine triphosphate

(Fahey et al. 2001; Raybould and Moyes 2001; Redovnikovic et al. 2008; Hopkins et al. 2009; Vig et al. 2009). However, these compounds have been identified in some plants of *Euphorbiaceae* family (Rodman 1991a, b; Rodman et al. 1998; Hu et al. 2010). More than 120 glucosinolated have been identified in the *Brassicaceae*. Some of them and their degradation products, such as sulphoraphane, crambene and indole-3-carbinol, are in the centre of interest due to their chemoprotective effect, especially in connection with malignant diseases (Clarke et al. 2011;

Hamsa et al. 2011; Lubet et al. 2011; Malaguti et al. 2011; Rajendran et al. 2011; Razis et al. 2011; Sharma et al. 2011; Shimamoto et al. 2011; Starrett and Blake 2011; Wiczk and Herman-Antosiewicz 2011). Their structure is based on ά-D-glucopyranose residue, which is linked via a sulphur atom to a (Z)-*N*-hydroximinosulphate ester. Basic structure is modified by variable R group, which is derived from some amino acids. In addition, it seems that sulphur in the glucosinolate structure may be replaced by similar selenium under selenogluco-sinolates formation. However, these compounds are still discussed (Bertelsen et al. 1988). Glucosinolates are derived from a limited group of amino acids, including alanine, leucine, methionine, phenylalanine and tryptophan. Their biosynthesis may be divided into three phases: (i) elongation of amino acid chain, in which methylene groups are inserted into the side chain, (ii) conversion of the amino acid moiety to the glucosinolate structure, and (iii) modification of the side chain – oxidation, hydroxylation, or esterification (Du et al. 1997; Ludwig-Muller et al. 1999; de Quiros et al. 2000; Graser et al. 2001; Falk et al. 2004; Field et al. 2004; Hirai et al. 2007; Sawada et al. 2007). However, their biosynthesis is still under the discussion Glucosinolates are stored in the vacuoles. After damage of plant tissue and consec-utive exposure to myrosinases, ά-thioglucoside glucosidases, they are hydrolysed to isothiocyanates and other products of toxicological importance, such as nitriles and thiocyanates (Xue et al. 1995; Rask et al. 2000; Andersson et al. 2009). Formation of next product is connected with protein factors called specifier proteins, which are able to promote formation of alternative products on the dependence on the glucosinolate side chain structure (Wittstock and Burow 2007; Burow and Wittstock 2009; Kissen and Bones 2009). Glucosinolates as well as the products of their hydrolysis are connected with the protection against herbivores and pathogens. Glucosinolates as well as product of their hydrolysis induce GST activity in some insect, such as *Myzus persicae* (green peach aphid) feeding *Brassica napus* and next members of the *Brassicaceae* (Vanhaelen et al. 2001; Francis et al. 2005). Defensive role for indole glucosinolates is suggested by the observation that atr1D mutant *A. thaliana* plants, which overproduce indole glucosinolates, are more resistant to *M. persicae*, whereas cyp79B2/cyp79B3 *A. thaliana* double mutants that lack indole glucosinolates, succumb to *M. persicae* more rapidly. Indole glucosinolate breakdown products, including conjugates formed with ascorbate, glutathione and amino acids, are elevated in the honeydew of *M. persicae* feeding from atr1D mutant plants, but are absent when the aphids are feeding on cyp79B2 cyp79B3 double mutants (Hanley and Parsley 1990; Kim et al. 2008).

On the other hand, response of *A. thaliana* to herbivore includes activation of genes involved in oxidative stress, calcium-dependent signalling and pathogenesis-related responses (Moran et al. 2002). Glutathione S-transferases (GST) are mainly cytosolic enzymes that catalyse the conjugation of electrophile molecules with reduced glutathione under formation of less toxic products. Enhanced GST activity was demonstrated in *Episyrphus balteatus* (hoverfly), and *Brevicoryne brassicae* (cabbage aphid). Isothiocyanates as degradation product of glucosinolates were detected in cabbage aphid (Chaplin-Kramer et al. 2011; Khan et al. 2011).
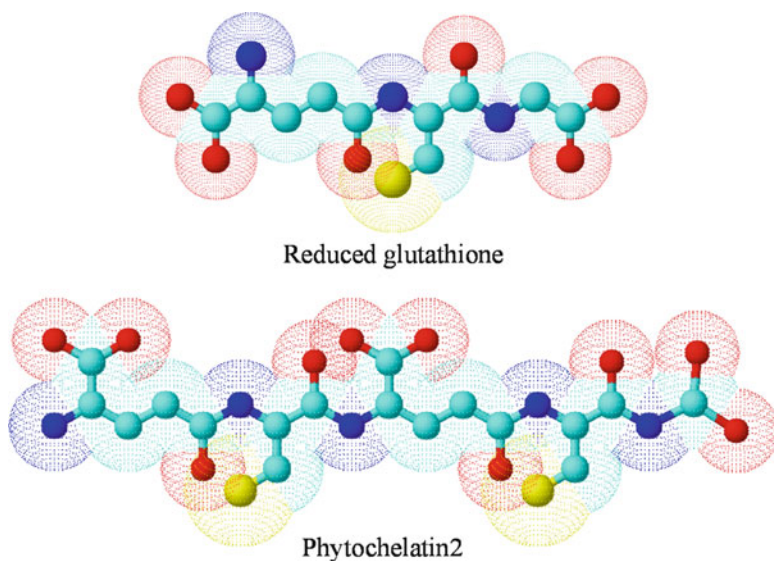
Fig. 3.4 3-D structures of reduced glutathione and phytochelatin2

Nevertheless, this chapter is not focused on biochemistry of glucosinolates and characterization of their breakdown product. For additional information, see published articles (Kim et al. 2008).

In plants, toxic substances become more water soluble (more hydrophilic) and they can subsequently be stored in vacuoles. Compartmentation in vacuoles plays important role in detoxification of many compounds. Tripeptide glutathione as well as phytochelatins as glutathione oligomers play crucial role in detoxification of heavy metal ions and xenobiotics in plants. Cysteine moiety/moieties with sulfhydryl (thiol) groups, which serves as a proton donor, plays essential role in their function.

Glutathione is synthesized from the amino acids L-cysteine, L-glutamic acid and L-glycine (Fig. 3.4). In the first step, γ-glutamylcysteine is synthesized from L-glutamine and L-cysteine via the enzyme γ-glutamylcysteine synthetase (glutamate cysteine ligase, GCL), in the second step, glycine is added to the C-terminal of γ-glutamylcysteine under glutathione formation. This reaction is catalysed by glutathione synthetase (GS). Phytochelatins are synthesized via phytochelatin synthases (PCS) activity (Fig. 3.4). There are many questions in the connection between glutathione and phytochelatins and glucosinolates. However, this relationship can be expected. Treatment of *A. thaliana* by cadmium ions led to the decrease of a content of total glucosinolates, especially indole glucosinolates (De Mortel et al. 2008; Sun et al. 2009). Similar results have been obtained in *Thlaspi praecox* and *T. arvense*. In the case of cadmium-sensitive *T. arvense*, its application led to the shift from alkenyl glucosinolates (mainly sinigrin) to indolyl glucosinolates. Treatment of the second species, *T. praecox*,

by cadmium led to the increase of total glucosinolates, especially benzyl glucosinolate sinalbin (Tolra et al. 2006). Connection between glucosinolates biosynthesis, zinc accumulation and organ specificity was demonstrated on *T. coerulescens* in the study of Tolra et al. (2001). Whereas zinc treatment led to the decrease of glucosinolate sinalbin in shoots, its concentrations increased with zinc accumulation. Changes in sinalbin were the most significant, other glucosinolates remained almost unaltered. Only week decrease of indolyl glucosinolates in both shoots and roots and 3-butenylglucosinolate in the roots was observed (Tolra et al. 2001). There are two fundamental questions. Where is a source of sulphur in glucosinolate biosynthesis? Firstly, differences have been observed in the processes of sulphur assimilation. Adenosine 5′-phosphosulphate (APS) kinase limits the availability of sulphur for biosynthesis of sulphur-containing secondary metabolites. Reduction of its activity leads to the reduction of glucosinolates biosynthesis and increase of thiol compounds formation in *A. thaliana*. Overexpression of enzyme APS reductase has no effect on glucosinolates production, but increases production of thiol compounds (Mugford et al. 2011). However, the source of reduced sulphur atom in the core glucosinolate structure remains unknown. Recent evidences show the possible role of glutathione (GSH) in this process. Work of Geu-Flores shows role of GSH as a sulphur donor in glucosinolates biosynthesis. The *A. thaliana* pad2-1 mutant is connected with reduced levels of glucosinolates, respectively camalexin. PAD2 gene encodes a g-glutamylcysteine synthetase that is involved in glutathione biosynthesis, so, pad2-1 mutant contains only 20% of the GSH in comparison with wild plants. Reduced GSH levels correlate with reduction of accumulation of the two major glucosinolates in *A. thaliana* – indolyl-3-methyl-glucosinolate and 4-methylsulfinylbutyl-glucosinolate (Schlaeppi et al. 2008). All these data suggest participation of GSH in glucosinolate biosynthesis and close connection between metabolism of thiol compounds and glucosinolates. The second question consists in involvement of enzymes connected with thiol biosynthesis in the biosynthesis of glucosinolates. Experiments with *A. thaliana* mutants impaired in the production of the γ-glutamyl peptidases GGP1 and GGP3 have demonstrated altered biosynthesis of glucosinolates. GGPs catalyse transfer of γ-glutamyl functional groups of glutathione to acceptors. These peptidases have been found in many plant species and are connected with many physiological processes in plants, including ripening of seeds/fruits and degradation of some biomolecules (Lancaster and Shaw 1994; Shaw et al. 2005; Li et al. 2008; Ohkama-Ohtsu et al. 2008). Function of known cytosolic γ-glutamyl peptidases in the processes of formation of GSH conjugates has been demonstrated in *A. thaliana* plants (Geu-Flores et al. 2011). Role of GG1 was investigated in benzylglucosinolate-producing non-cruciferous plant *Nicotiana benthamiana*. In this study, increased levels of benzylglucosinolate in the presence of GGP1 were accompanied by the high accumulation of the last intermediate desulfobenzylglucosinolate. This fact suggest role of sufotransferase AtSOT16 in the formation of terminal product – benzylglucosinolate (Moldrup et al. 2011). Role of PCS in glucosinolate biosynthesis is widely discussed (Clay et al. 2009). It seems that

PCS serve as a peptidase in indole glucosinolate biosynthesis in *A. thaliana*. Glucosinolate-derived molecules seem to be the most likely candidates as PCS substrates in *A. thaliana* (Clemens and Persoh 2009). All these data suggest role of PCS not only in homeostasis of heavy metals, but also their regulatory role in glucosinolates biosynthesis.

3.7 Impact of Members of *Brassicaceae* Family on Cadmium Elimination from Living Environment – Possibilities of Increasing of Cadmium-Hyperaccumulation by *Brasicaceaeans*

There are different strategies in enhancement of accumulation properties of members of the *Brassicaceae*. The first approach consists in supplementation of soil by additives, compounds that are able to increase uptake of heavy metals including cadmium. There are many compounds, which were tested of possible chelators of heavy metals. However, their usage has many disadvantages including potential toxicity with reduction of biomass production and hazard for the environment due to mobilization of heavy metals. On the other hand, heavy-metals accumulating plants may represent potential risk because of their possible entry the food chain. All these questions must be carefully considered. Chelators in the phytoremediation by the *Brassicaceae* are summarized in Table 3.3.

In the *Brassicaceae*, effect of two different compounds on cadmium uptake – nitrilotriacetate (NTA) and citric acid (CA) – by Indian mustard (*Brassica juncea*)

Table 3.3 Chelators used in members of the *Brassicaceae* in phytoremediation

Chelator	Heavy metal(s)	Plant species	Reference(s)
EDTA, EDDS, histidine	Cu, Pg, Fe, Zn	*Brassica juncea*	Karczewska et al. (2009)
EDTA	Cd, Cr, Ni	*Thlaspi caerulascens*	Munn et al. (2008)
EDTA, DTPA Oxalic acid, citric acid	Cr, Ni	*Brassica juncea*	Hsiao et al. (2007)
EDTA	Cu, Cd, Pb, Zn	*Brassica napus* *Brassica juncea*	Turan and Bringu (2007)
Nitrilotriacetate Citric acid	Cd	*Brassica juncea*	Quartacci et al. (2005)
[S,S]-ethylenediamine disuccinate	Pb	*Thlaspi goesingense*	Finzgar et al. (2005, 2006)
Thiol-rich chelators	As, Hg	*Arabidopsis halleri*	Meagher and Heaton (2005)
EDTA, EDDS, DTPA	Cu	*Brassica rapa* v. *pekinensis*	Kos and Lestan (2004)
NH$_4$Cl, casein, citric acid	Cd, Cu, Ni, Zn	*Brassica chinensis*	Gramss et al. (2004)
EDTA	Pb	*Brassica juncea*	Blaylock et al. (1997)

were investigated in the study of Quartacci et al. (2005). NTA-treated plants demonstrated twofold increase of cadmium accumulation compared to control plants (Quartacci et al. 2005). EDTA, EDDS and histidine as potential chelators of Cu, Pb, Fe and Zn for *Brassica juncea* uptake were used in experiments of Karczewska et al. (2009). Synthetic aminopolycarboxylic acids ethylenediamine tetracarboxylic acid (EDTA) and diethylenetriamin pentaacetate (DTPA) together with low-molecular organic acids oxalic acid and citric acid were used in experiments with *Brassica juncea* cultivated in serpentine-mine tailings contaminated by Cr and Ni. Authors declare advantages of the use of low-molecular organic acids as chelators due to reduction of environmental risk in phytoremediation (Hsiao et al. 2007). However, further data are still missing.

The second approach is based on construction of transgenic plants. This method is based on an introducing of foreign genes, which are connected with uptake, transport and accumulation of heavy metal/metals. A yeast cadmium factor 1 (YCF1), a member of the ATP-binding cassette (ABC) transporters, is localised at the vacuolar membrane in *Saccharomyces cerevisiae*. YCF1 gene introduced to the transgenic *Brassica juncea* plants showed 1.3- to 1.6-fold tolerance to cadmium ions compared to wild plants (Bhuiyan et al. 2011). Overexpression of BjCdR15 in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* led to the enhancement of cadmium tolerance. *Arabidopsis* tga3-2 mutants demonstrated high cadmium accumulation in roots and inhibition of its transport into aerial parts (Farinati et al. 2010). Possibilities of different approaches including genetic manipulations for improvement of heavy metals tolerance and accumulation are summarized in reviews (Pilon-Smits and Pilon 2002; Singh et al. 2003; Czako et al. 2006; Lynch 2007; Yadav 2010; Anjum et al. 2012).

3.8   Conclusions and Perspectives

An ideal plant for phytoremediation should meet several criteria: (i) to grow rapidly and to produce large quantities of biomass, (ii) to deeply root and to have an easily harvested shoots, and (iii) to accumulate high concentrations of contaminants in the shoots. Presently known hyperaccumulators of metals, in other words plants capable of over-accumulation metals in their tissues, meet the third criterion of an ideal plant for phytoremediation as *Brassicaceae*. In contrast, there other plants as crops meet the first two criteria of an ideal plant for phytoremediation. The combination of these properties by some genetic manipulation could be very promising for the future of remediation of polluted environment by plants.

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