# Chapter 8
# Initial Steps of Copper Detoxification: Outside and Inside of the Plant Cell

**Valentina P. Kholodova, Elena M. Ivanova, and Vladimir V. Kuznetsov**

## 8.1 Introduction

Increasingly high technogenic load on the environment raises a problem of strategies and mechanisms of plant adaptation to abiotic stressors, among which heavy metals (HM) occupy a specific place. Contamination of vast territories with HM acquires more and more threatening character.

According to their toxicity, all HM are arranged in the so-called Irving–Williams series, where Cu takes the first place corresponding to the highest level of toxicity (Krämer et al. 2007; Yruela 2009). Copper (Cu), being the most toxic metal, at the same time belongs to so-called essential elements. This term designates the group of HM, which trace amounts are required for plant metabolism, growth, and development, whereas their high concentrations are toxic (Hall and Williams 2003).

Under physiological conditions, copper occurs in the cell in reduced ($Cu^{+}$) and oxidized ($Cu^{2+}$) states. Being a protein cofactor, copper is involved in the processes of photosynthesis and respiration, in the perception of the ethylene signal, in plant protection against oxidative stress, in the biogenesis of molybdenum cofactor, and also in the cell wall metabolism (Yruela 2009). However, the range of Cu concentrations suitable for the optimum cell metabolism and plant development is rather narrow. It is believed that even twofold exceeding of these concentrations could exert harmful effects, whereas the higher Cu concentrations induce a toxicity syndrome (chlorosis and necrosis, stunting, inhibition of root and shoot growth) and in most cases result in plant death. An exclusion from this rule is metallophytes highly tolerant to Cu, but their number is small.

At the cellular level, Cu and other HM toxicity is determined by (1) binding to SH groups in proteins, thereby inhibiting enzyme activity or protein function;

V.P. Kholodova (✉) • E.M. Ivanova • V.V. Kuznetsov
Laboratory of Physiological and Molecular Mechanisms of Adaptation, Timiryazev Institute of Plant Physiology RAS, Botanicheskaya ul., 35, Moscow 127276, Russia
e-mail: v-p-kholodova@ippras.ru

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(2) induction of deficiency of other essential ions; (3) impaired cell transport processes; and (4) oxidative damage (Yruela 2009; Cohu and Pilon 2010).

Soil contamination with Cu occurs mainly on territories adjacent to the zones of copper ore deposits close to the surface of the earth, in its mining and processing sites, and near numerous industrial plants. Another source of large-scale soil contamination is agricultural industry: copper entries into soil with fertilizers or plant defense preparations. Such soil contamination is especially characteristic for vineyard where multiyear plant treatment with Cu-containing preparations results in a sharp increase in its content in soil. At the usual average Cu concentration in soil of 20–30 mg/kg, its content in contaminated soil could exceed these values in tens and hundreds times and attain 250–1,000 mg/kg of soil (Krämer and Clemens 2006; Yruela 2009).

In this connection, the question raises how plants control Cu homeostasis under conditions of its excess in environment, e.g., how they can provide the cells of all tissues and organs with Cu micro amounts required to sustain life and, on the other hand, prevent its toxic effects resulting in plant death.

It should be noted that Cu is really present in all cellular structures: nucleus, mitochondria, chloroplasts, Golgi apparatus, and endoplasmic reticulum. This is achieved due to functioning of several families of more or less specialized membrane transporters localized in the plasma membrane and all other cell membranes. However, at a great Cu excess in environment, its transport into the cells is performed also by unspecialized transporters, which transport other two- and/or monovalent ions across the membranes as well. Fine regulation of transporter and metallochaperon functioning provides all organism cells with Cu micro amounts.

Our task was to study the mechanisms underlying plant protection against toxicity of high Cu concentrations in soil. Furthermore, it is established that the intracellular concentration of free Cu ions does not exceed $10^{-21}$–$10^{-18}$ M, i.e., one ion per cell (Changela et al. 2003). This indicates that a rather efficient system of Cu ion chelating operates in the cell cytosol. In this process, an important role belongs to highly specialized chelators, phytochelatins and metallothioneins, which function in coordination with another group of proteins, metallochaperons. Metallochaperons provide for delivering of Cu–ligand complexes to the sites of Cu destination. It is evidently true for both transport of Cu micro amounts to the sites of their functioning in corresponding macromolecules and transfer of excessive chelated Cu ions penetrated into the cytosol to the sites of their detoxification. Thus, the main components of the intracellular defense system against Cu excess are chelators, metallochaperons, and, naturally, membrane transporters (Lee et al. 2006; Mari and Lebrun 2006; Yruela 2009). In spite of rather wide-ranging studying of HM excess chelating in the cytosol of plant cells, the contribution of chelators and metallothioneins in Cu detoxification remains rather debatable (Gonzalez-Mendoza et al. 2007; Guo et al. 2008; Yruela 2009).

The problem of Cu ion detoxification in the apoplast is poorly studied, although just the apoplast is the first site of the plant cell contact with Cu excess in environment and evidently it could be considered one of the important barriers on the route of excessive HM penetration into the cytosol. We would call the apoplast

all compartments beyond the plasma membrane (Sattlemacher 2001), although the main attention would be paid to the interfibrillar and intermicellar spaces of the cell walls. However, before discussing the role of the apoplast per se in Cu detoxification, we would recede still farther from the tissue symplast pool of Cu and analyze briefly the role of arbuscular mycorrhizal fungi (AMF), which colonize the root cortex of most higher plants and produce an extraradical mycelium (Joner et al. 2000; Ferrol et al. 2009), i.e., we consider events occurring in the rhizosphere and appraise the role of these structures in Cu ion immobilization.

## 8.2 Role of Arbuscular Mycorrhiza

AMF are obligate biotrophs of higher plants. They colonize the root cortex of most higher plants and develop an extraradical mycelium, which grow in soil around the roots (Ferrol et al. 2009). Mycorrhizal fungi constitute the only group of microorganisms that are capable of transporting mineral elements from the soil solution to plants (Joner et al. 2000). By now, it is well established that symbiotic combination of metallophytes with AMF could enhance plant ability to grow on highly contaminated soils (see review by Hildebrandt et al. 2007). One of the main reasons for activation of plant growth in such symbiotic systems is believed to be the improved plant supply with phosphorus and nitrogen (Ferrol et al. 2009).

May be this was the reason for a protective effect of AMF *Glomus mosseae*, which was clearly manifested in three from four tested plant species (Chen et al. 2007). In *Trifolium repens, Coreopsis drummondi*, and *Pteris vitata* (fern), the root length and the root and shoot dry weights were substantially increased at 14–18% root colonization with *G. mosseae*. AMF did not essentially affect the concentration of Cu in the roots but markedly reduced it in the shoots. However, since the shoot biomass was strongly increased, total Cu amount in shoots, as calculated per plant (or vessel), was increased by 4.7 times in *T. repens*, 4.9 times in *C. drummondii*, and 8.13 times in *P. vitata*. The authors did not analyze the reasons for such AMF effects on plants, considering a "dilution effect" as the key mechanism of AMF-mediated diminishing of Cu (and Cd) concentration in aboveground organs.

At the same time, another hypothesis of AMF protective action is more popular. According to this hypothesis, AMF, which colonize plant roots, considerably reduce the uptake of HM into plant cells, and this may be one of the mechanisms of metallophyte tolerance to HM (Hildebrandt et al. 2007). Nevertheless, the realization of this mechanism is evidently species specific.

This suggestion was supported by the experiments of Sudova et al. (2008), who failed to establish a synergism between *Agrostis capillaries* and AMF *Glomus intraradices*, although these authors used various clones and isolates originated either from contaminated or uncontaminated sites. It turned out that AMF did not confer significant additional Cu tolerance to either Cu-tolerant or Cu-sensitive host plants grown on the contaminated substrate, even in the case of highly Cu-sensitive clones. Nevertheless, the authors did not exclude a possibility that beneficial

interaction between the two organisms might be manifested for other combinations of plant species and fungal species/isolates.

Another situation was observed at inoculation of the salt marsh plant *Aster tripolium* with inoculates of AMF *Glomus geosporum* originated from polluted salt marshes (PL isolates) or from nonpolluted sites (NP isolates) (Carvalho et al. 2006). In the presence of high Cu concentrations in soil (up to 2.0 mM), tolerance of nonmycorrhizal (NM) plants was relatively high: the dry weight of their roots and shoots exceeded corresponding indices for PL and NP plants. At the same time, the content of Cu in the roots of PL and NP plants was twice higher that in the roots of NM plants, whereas, in contrast, the shoots of NM plants contained the highest amount of Cu. The authors concluded that AMF enhanced Cu uptake and accumulation in the root system but retarded its translocation to shoots, as compared to NM plants. They called this effect "toxic metal trapping."

*Oryza sativa* colonization by the extraradical mycelium of AMF *G. mosseae* resulted in the stronger fungal effects (Zhang et al. 2009). When colonized plants (GM plants) were grown in the presence of 5–100 μM $CuCl_2$, Cu contents in their roots and especially in their shoots were significantly reduced. Thus, the content of Cu in the shoots was only 48% of that in NM plants. In this case, more than 60% of Cu present in the GM plants was retained in the root cell walls, whereas the corresponding value in NM plants was only 25%. The authors supposed that the reason for this difference is some changes they observed in the composition of the GM plant cell walls and, thus, in their Cu-binding capacity, i.e., cation exchange capacity (CEC).

Gonzalez-Chavez et al. (2002) evaluated directly the role of CEC for extraradical mycelium of three *Glomus* species colonizing *Sorgum vulgare*. Isolates of all species were obtained from polluted soil and one of them from nonpolluted soil as well. Observation performed with the usage of TEM and SEM linked to an energy dispersive X-ray spectrometer (EDAX) showed that AMF from polluted soil were able to accumulate Cu in different zones of their extraradical mycelium, in mucilaginous outer hyphal wall zone, cell walls, and inside the hyphal cytoplasm. The difference between the three AMF in the content of Cu accumulated in the extraradical mycelium was evidently primarily (might be partly) related to their difference in CEC. Metal ion filtering during their uptake by the roots might be one of important protective AMF functions in Cu-contaminated sites.

Such AMF role was confirmed in several researches. Thus, in the work of González-Guerrero et al. (2008), it was shown using EDXS that Cu accumulated mainly in the fungal cell walls and in the electron-dense granules in the vacuoles of fungal (*Glomus intraradices*) spores but not in the cytoplasm. A comparison of CEC of *Trifolium subterraneum* roots and extraradical hyphae of several *Glomus* species (Joner et al. 2000) showed that fungal hyphae manifested approximately tenfold higher CEC than roots, and there was no difference between different fungal species.

Thus, investigations on the effects of excessive Cu concentrations on the symbiotic plant-AMF systems showed clearly a diversity of mechanisms used at plant fungal colonization for diminishing Cu excess toxicity. Along with sometimes

observed dilution, such a mechanism may be Cu entrapping or filtering resulting in retaining the great part of Cu excess in the mycelium and, as a consequence, in observed decrease in the Cu content within the plant and its translocation from roots to shoots. It is quite possible that the main reason for this effect is a high CEC value of AMF mycelium (toward Cu), which exceeds markedly CEC of colonized plant roots. In this connection, the data concerning the involvement of insoluble glycoprotein, glomalin, in Cu detoxification are of a great interest. In the experiments performed in vitro, this protein could sequester up to 28 mg Cu/g of protein (González-Chávez et al. 2004). For the appraisal of the cell wall protective role, the fact (established with monoclonal antibody MAb32B11) that glomalin is mainly located in the cell walls of AMF hyphae is of especial importance (Purin and Rillig 2008; Ferrol et al. 2009).

At the same time, data concerning changes in the cell wall composition of roots colonized by AMF (Zhang et al. 2009) deserve an earnest consideration. Such changes may be the reason for Cu retaining in the root tissues; they indicate a possibility of direct influence of fungal mycelium on the metabolic processes in the host plant tissues. This suggestion is confirmed by studies performed in the laboratory of Hildebrandt (see review Hildebrandt et al. 2007); it was shown that, in the symbiotic system, the HM, including Cu, excess not only activated markedly the defense system of the mycelium but also induced complex and ambiguous changes in expression of plant genes encoding products putatively involved in HM detoxification.

## 8.3 Apoplast Involvement in Copper Detoxification

Copper penetrates into the plant mainly as a bivalent cation $Cu^{2+}$, although it was reported that sometimes it is reduced near the root surface and penetrates into the root cells as a monovalent cation (Krämer and Clemens 2006; Cohu and Pilon 2010) or even as a free metal (see Sect. 8.5.2). Since the cell wall is a negatively charged ion exchanger, it is quite clear that a part of Cu ions is absorbed by the cell wall on the path of their movement into the cell. Moreover, an additional copper amount may be released into the apoplast from symplast because transporters of the $P_{1b}$-ATPase family, denoted as heavy metal ATPase 5 (HMA5), are located in the plasma membrane and provide for the efflux into the apoplast of excess Cu ions, which have previously penetrated inside the cell. The important role of apoplast in Cu detoxification was supported by experiments of Andres-Colas et al. (2006), who demonstrated that the *hma5* T-DNA insertion mutant exhibited Cu hypersensitivity, which was especially dramatic in roots where *HMA5* was mostly expressed. These data allow a consideration of HMA5 P-type ATPase functioning as a potential mechanism for improving Cu detoxification under Cu excess.

Cell walls of different plant species differ substantially in their composition (Carpita et al. 2001). Negatively charged groups of the polymeric matrix play a key role in binding of HM, including Cu ions. The high content of pectic compounds

containing unetherified carboxyl groups of polygalacturonic acid plays a crucial role in this process. The content and qualitative composition of phenolic compounds and lignin also exert substantial influence.

Strong differences in the composition and properties of the cell walls in different plant species are evident; they depend on plant age, environmental cues, and other factors. CEC of polymers in the cell wall matrix is one of the main parameters determining ion exchange in them (Meychik and Yermakov 2001; Wehr et al. 2010). This parameter is frequently assessed on isolated, chemically purified and, thus, to some degree modified cell walls. This raises a question as to whether degree the obtained results correspond to the natural state of the cell walls before their isolation. Microscopic methods offer a closer approach to the evaluation of the natural cell wall capacity for HM immobilization. However, the methods of direct Cu visualization with the usage of specific reagents are not essentially developed, excluding few studies (Arru et al. 2002; Ranathunge et al. 2005). The methods of electron microscopy present great possibilities; the combination of TEM or SEM with modern physical methods, EDX or EELS, makes a reality the quantification of Cu intracellular localization in plants.

### 8.3.1 *Microscopic Methods of Cu Localization in the Cell Wall and Periplasmatic Space*

Using TEM, Cu was detected in the roots of *Zea mays* (Ouzounidou et al. 1995) and *Oreganum vulgare*, a plant of the Mediterranean aromatic flora (Panou-Filotheou and Bosabalidis 2004) grown under Cu great excess in medium. On TEM micrographs, Cu was seen as electron-dense globular inclusions, near the cell walls in particular. Cu was deposited in the cell walls, including their middle-lamellar regions. In addition, dense compact material was located behind the plasmalemma, adjacent to the walls in cortical and stellar parenchyma cells.

Copper localization in leaf and stem tissues of *Cannabis sativa* plants, grown for 10 days on medium supplemented with 1 mM $CuSO_4$ (100-fold above normal concentration) but without any visible toxicity symptoms, was studied after Cu immobilization with $Na_2S$ (Arru et al. 2002). In these TEM experiments, electron-dense deposits were observed in vacuoles and also as very fine precipitates at the cell junctions, mainly on the middle lamella. The presence of Cu in these structures was confirmed by EDX-ray microanalysis in the epidermis and in leaf palisade layers. The authors concluded that, in *C. sativa* leaves, Cu ions followed symplastic as well as apoplastic route.

Structural changes, induced by the excess of Cu (10 μM) in the photosynthesizing suspension cell culture of *Glycine max,* were studied (Bernal et al. 2006). Using LM, numerous dark rounded deposits were observed attached at the outer surface of the cell wall. These extracellular dark deposits of different sizes and shapes (round and ellipsoid), contacting with the cell walls, contained Cu, and this was confirmed by EDX-ray microanalysis. The authors noted that such structures were present only in

Cu-stressed cells (21 days on medium with 10 μM $CuSO_4$) but not in control treatment or after long-term acclimation (after 22 subculturings in the presence of 10 μM Cu).

A comprehensive study was performed on *Armeria maritima* plants growing on the copper-rich soil, which accumulated from 2,000 (leaves) to 4,000 (roots) higher amounts of Cu as compared with its standard concentrations in plants (Neumann et al. 1995). As measured by TEM-EDX analysis, a great part of Cu in roots and leaves was retained in vacuoles of idioblasts ("tannin cells"). Similar osmiophilic precipitates (with high Cu concentrations) were shown between the cell wall and plasmalemma and in the cell wall of root cortical parenchyma. Copper contents in cell walls of the exodermis (in roots) and idioblast/bundle (in leaves) of *A. maritima* were especially high. EELS spectra of cell-wall-bound Cu showed that Cu was evidently bound in the protein–copper complexes. Two copper-binding proteins were extracted from the cell walls.

Another species, *Elsholtzia splendens*, a native Chinese Cu-tolerant and Cu-accumulating plant, was studied by Peng et al. (2005). Using TEM, these authors observed in the leaves large amounts of Cu, which were intensively deposited both within the cells (in the chloroplast membranes) and in the cell walls. Still larger and numerous big dark Cu particles were noted in the root cells, especially at the high $CuSO_4$ concentration in medium. These particles were deposited near the root cell walls and plasma membrane. Numerous Cu deposits separated the cytoplasm and the cell wall. It should be noted that such numerous large Cu deposits were found in this work in spite of the very high Fe–EDTA concentration (100 μM) in medium and 20-min root washing with 5 mM $Pb(NO_3)_2$ before fixation. These researchers also presented quantitative Cu estimates for isolated cell walls obtained by the gradient centrifugation technique (see Sect. 8.3.3).

Quantification of Cu distribution between root and leaf tissues of *Avicennia marina* plants, a facultative halophyte and a typical mangrove species, was performed using SEM X-ray microanalysis in the work of MacFarlane and Burchett (2000). Because of method limitations, a very high $CuSO_4$ concentration (4 g/l) in the substrate was applied, but the time of exposure was shortened to 4 days to minimize the onset of Cu toxicity responses. In the root zone of developed endodermis with Casparian bands, the relative Cu concentration was similar from epidermal through parenchyma to the endodermal cell walls, the lowest concentration being in the phloem. Cell wall Cu concentrations were higher than cytoplasmic ones; the difference was small in epidermis and parenchyma cells and significantly higher (by ~30%) in the endodermal cell walls. In the leaf tissues, the highest concentration was found in the xylem cell walls; it decreased gradually with an increased distance from the xylem; in the mesophyll cells, Cu content in the cell walls was higher than in the cytoplasm.

As distinct from above data, in experiments with *Allium sativum* plants grown in the presence of high Cu concentrations (10 and 100 μM) in nutrient medium (and 1 μM Cu in control treatment), only trace amounts of Cu were detected in the cell walls after 9-day-long exposure (Liu and Kottke 2004). The TEM-EELS analysis showed that the main amount of Cu was precipitated in the cell walls of cortical

cells. The authors believe that the difference of their results from those of other researchers is determined by species specificity. One more cause may be a rather high EDTA concentration (10 μM Fe–EDTA) in medium, which could limit Cu availability for the roots. A decrease in the free copper ion concentration in the near-root medium can be a reason for the diminished Cu ion absorption by the cell walls.

Thus, the results of microscopic investigations are mainly qualitative. Nevertheless, in various plant species belonging to various ecological groups, Cu precipitates, usually numerous globular structures, were observed in the cell walls, mainly on the middle lamellae and also in the closed intercellular space restricted by the cell walls. It should be emphasized that Cu-containing globules were also detected near the cell wall, in the periplasmatic space, between the cell wall and plasma membrane (Neumann et al. 1995; Panou-Filotheou and Bosabalidis 2004; Peng et al. 2005; Bernal et al. 2006). The presence of Cu in this space in its insoluble form (small precipitates, clumps, globules, etc.) could be evidently explained by the ways of its immobilization in this small volume: it seems likely that Cu was retained in the periplasmatic space by other compounds than the components of the cell wall.

### *8.3.2 Some Approaches for Distinction Between Apoplastic and Symplastic Cu Pools*

To quantify Cu distribution between its main tissue pools, i.e., between the apoplast and symplast, it is of importance to measure the CEC of the cell walls. This parameter characterizes the process of cation exchange and a possibility of Cu immobilization by the cell walls (Meychik et al. 2010). The main way to estimate CEC is a correct performing of metal (Cu) desorption using Cu-displacing agents, which could replace it from the cell wall matrix, with subsequent determination of Cu content by any one of methods, most often spectrophotometrically. This approach is now applied most frequently, although, regretfully, the common methodological version of the assessment is not elaborated. Only few works analyze the correctness of the approaches applied.

One of the first studies of copper distribution between the apoplast and symplast was that of Harrison et al. (1979). The authors screened several metal cations on their relative capacity for Cu desorption from the free space of *Hordeum* roots. Such desorption is difficult because of especially high stability of copper complexes with charged sites of the cell wall matrix. After testing the collection of metals (Pb, Ca, Cd, Co, Mg, and Ni), the authors established that Pb was most suitable as a Cu-displacing agent. The efficiency of 5 mM $Pb(NO_3)_2$ (60 min at 0°C) relative the wide range of Cu concentrations was confirmed by the authors in both time-course and kinetic studies.

Rather few early investigations using $Pb(NO_3)_2$ as a desorbent were reviewed by Ernst et al. (1992). The authors concluded that the amount of metals bound to the cell wall was usually less than 10% of its total cellular amount. As to the role of cell

walls in HM detoxification, both positive correlation between HM tolerance and cell wall binding capacity and the absence of such correlation were observed.

Later, Pb was repeatedly used as an effective agent for apoplastic Cu displacing in the roots and leaves of many plant species (De Vos et al. 1991; Yang et al. 2002; Llugany et al. 2003; Peng et al. 2005; Russo et al. 2008; and others). In most cases, the role of this cellular Cu pool was analyzed.

In recent years, short washing with 3 mM EDTA came to be applied to remove free cations. This approach was, for example, used for soybean suspension culture and leaves (Bernal et al. 2006, 2007) and Arabidopsis plants (Andres-Colas et al. 2006). Branquinho et al. (1997) compared carefully several ways of Cu desorption, including EDTA and Pb application, for several lichen species. They showed that elution procedure with 20 mM $Na_2$–EDTA (a chelating agent) (pH 4.5, sequentially for 40 and 20 min) was as efficient as that with 20 mM $Pb(NO_3)_2$. In contrast, the usage of $NiCl_2$ for Cu desorption resulted in the underestimation of the apoplastic Cu pool. It is of importance that EDTA and Pb did not disturb plasma membrane integrity, as evident from the absence of the effects of these agents on the content of intracellular potassium. In authors' opinion, this might be a cause for great errors. The efficiency of $Na_2$–EDTA usage for determination of the apoplastic Cu pool was confirmed in some studies (Monnet et al. 2005; Russo et al. 2008; see also the review of Bačkor and Loppi 2009).

However, in many studies, researchers applied only poorly controlled washing with distilled water. Such approach did not allow the assessment of Cu concentration within the cells, in the symplast. Application of some surfactants, such as Alconox or Triton in combination with EDTA, and lauryl sulfate were used mainly for cleansing the surface of aboveground organs from Cu contamination (Faucon et al. 2007, 2009). Only few publications comprise quantitative data concerning Cu content in the apoplast and its proportion in the total Cu content in tissues.

Branquinho et al. (1997) and Monnet et al. (2005) presented quantitative data obtained for intact lichens. Branquinho et al. (1997) used $Na_2$-EDTA as a displacing agent to assess extracellular bound Cu (see above) in *Usnea* spp. and *Ramalina fastigiata*. It was established that, in the presence of the highest Cu concentration in medium (15.8 mM $CuSO_4$), extracellular binding of Cu reached equilibrium after 10–20 min, and after 2 h, almost all Cu was immobilized by the cell walls, and not more than 2% in *Usnea* and about 5% in *R. fastigiata* was localized within the cells. For both species, the concentration dependence of extracellular Cu binding showed saturation kinetics, and the maximum capacity to bind extracellular Cu was approximately fivefold higher for *Usnea* spp. than for *R. fastigiata*.

Monnet et al. (2005) also used $Na_2$–EDTA as an extracellular Cu extractant from *Dermatocarpon luridum* thalli. They were able to determine and quantify the cellular location of Cu in lichens in the presence of 0.25–1.00 mM Cu in medium. At all Cu concentrations in medium, the extracellular Cu concentration reached a maximum value after 3–6 h, but a significant decrease was then observed in the presence of 0.25 or 0.50 mM $CuSO_4$. (It is worth mentioning that the first measurement was performed in this study only in 3 h after treatment.) Measurements

performed for 48 h allowed the authors to conclude that the extracellular content of Cu represented more than 90% of total Cu content.

A specific approach for separation of apoplastic and symplastic Cu pools was applied in the study of Zhang et al. (2009) performed on *O. sativa* plants, and this is evidently the only work of this sort for Cu. After Cu desorption from the roots with 5 mM $CaCl_2$ (four times, 5 min each), the roots were subjected to fast freezing in liquid nitrogen with subsequent thawing and rinsing with water. Cu released after such treatments was considered symplastic Cu. The total content of Cu released in the 20-min desorption and Cu remained in the root after the freeze–thaw cycle was considered apoplastic Cu. In these experiments, the highest value of apoplastic Cu was about 25% of its total content in the *O. sativa* roots not colonized by *G. mosseae*, but this proportion increased to 60% under conditions of mycorrhizal colonization, mainly due to a decrease in the total Cu content in plants.

Regretfully, only several studies comprised quantitative data characterizing the Cu apoplastic pool in intact plant organs. The investigations in this direction must be expanded. Correct methods for separation of apoplastic and symplastic Cu pools in plant organs should be elaborated. In our opinion, the principle of the method developed by Harrison et al. (1979) could be used for the quantitative assessment of Cu partition between the root apoplast and symplast, maybe after some modification. We performed some work in this direction.

The main principles of the Harrison et al. (1979) method are: (1) Cu uptake by excised roots to exclude its translocation from roots to shoots and (2) low temperature during replacement of the apoplast pool to prevent Cu transport from the apoplast (cell walls and periplasmatic space) to the symplast. In our method modification, all procedures, Cu uptake and displacing, were performed at low temperature (lower than 4°C).

To apply these methods, it was of importance to find a correct criterion for distinction between the pools. $Na_2$–EDTA was chosen as a displacing agent, whereas EDTA concentration and the time of desorption were variable parameters. When the values of these two parameters were increased to a definite limit, a sharp jump in Cu leakage from the tissue was observed. We consider such a jump as a sign of plasma membrane damage resulting in the mixing of two divided pools. Therefore, for separate quantification of these pools, we use conditions preceding this jump.

When Cu uptake occurred at a temperature close to zero, we can obtain direct information about apoplast filling because transmembrane Cu transfer into symplast, which would be performed by protein transporters under sufficient level of energy supply, is blocked by low temperature.

It is quite clear that possibilities of the approach we elaborated are rather limited. Indeed, for each plant species and experimental conditions, the limiting values of variable parameters and their correctness should be thoroughly checked. Undoubtedly, some additional modifications are required for wide application of this method.

The analysis of the Cu apoplastic pool, its physiological functions, and possible involvement in metal detoxification will be presented below together with the results obtained for isolated cell walls.

### 8.3.3 *Isolated Cell Walls as a Model for Studying Cu Immobilization in the Apoplast*

In some studies, CEC relative to Cu was studied on cell walls isolated from roots and/or leaves of plants, in particular plants differing in their tolerance to excessive Cu concentrations. The methods used for cell wall isolation differ substantially, and this hinders generalization of published works. Nevertheless, the basic information concerning this problem was obtained just on isolated cell walls.

In most works, cell walls were isolated using buffer solutions (pH 7.0–7.5) with corresponding additions for the removal of low-molecular soluble organic compounds (Nishizono et al. 1987; Konno et al. 2005; Peng et al. 2005; Fritioff and Greger 2006; Ke et al. 2007; Wehr et al. 2010). In the works of Lou et al. (2004), Shi et al. (2008), and Wei et al. (2008), before cell wall isolation, plant material was soaked in the mixture of methanol and chloroform (2:1, v/v). Zhang et al. (2009) applied a very specific method for cell wall isolation but also with the usage of organic solvents.

It turned out that a great part of studies were performed on isolated cell walls from Cu-tolerant and Cu-accumulating plants.

Several works were performed on the two species of Elsholtzia. Important information concerning the dynamics of apoplastic Cu pool accumulation was obtained for *Elsholtzia haichowensis*, a native Cu-tolerant Cu-accumulating plant species growing on copper mining deposits in China (Lou et al. 2004). The saturation of sites for Cu adsorption was achieved after 72 h exposure to the 100 μM $CuSO_4$ solution. During the first 24 h, most of the increased Cu in roots was found in the cell wall fraction. By the end of experiment (over 120 h), Cu bound to the cell walls accounted for 68% of the total Cu content in roots.

Ultrastructural Cu distribution in both the roots and aboveground organs was examined for another highly tolerant species, *E. splendens* (Peng et al. 2005; Shi et al. 2008). In these plants exposed to 500 μM Cu during 8 days, total Cu concentration in the leaves increased fivefold, up to 250 mg Cu/kg, whereas in the roots an increase was much greater, up to 8,000 mg Cu/kg (Peng et al. 2005). The authors calculated that 37% of total Cu in the leaves and 56% in the roots were accumulated in the cell walls. On this basis, it was concluded that in *E. splendens*, a Cu-tolerant and Cu-accumulating plant species, the plant cell wall was the main Cu location site both in the root and leaf cells. It could mainly account for the high detoxification of Cu in the plant.

In another work performed on the same plant species, *E. splendens,* subcellular distribution of Cu was analyzed by Cu K-edge XANES method (Shi et al. 2008). It was established that 30–39% of total Cu in the roots and stems and 20–44% in the leaves were localized in the cell walls. It turned out that, in the interval between 10- and 60-day-long plant exposures to 300 μM $CuSO_4$, Cu concentration in the roots increased only by 17%, whereas in the leaves, by 50%. Cu redistribution was manifested much more profoundly in the leaf cell walls (from 44 to 20%) as compared with the root cell walls (from 39 to 31%). At the same time, the content of Cu bound with histidine-like intracellular ligands was increased.

One of the earliest studies of intracellular Cu localization was that by Nishizono et al. (1987) performed on the fern *Athyrium yokoscense*, which is known in Japan as a highly HM-tolerant plant. The concentration of Cu in the roots of this plant growing on metalliferous soils exceeded this index in plants from nonmetalliferous habitats by many times (2,602–3,846 vs. 26–70 μg/g DW of roots). At the same time, the difference between relative Cu content in the root cell walls of these plants from different habitats was not too strong: 85–92% of its total content in plants growing on metalliferous soils vs. 72–76% in plants on nonmetalliferous soil.

The values of CEC for Cu, determined in the isolated *A. yokoscense* cell walls, were by 5–7 times higher than this index measured for several plant species growing in copper-contaminated areas. The CEC value was well correlated with the Cu concentration accumulated in *A. yokoscense* roots. The high values of cell wall CEC in *A. yokoscense* roots did not depend on the extent of habitat contamination. However, a difference between *A. yokoscense* and other fern species, nontolerant to Cu excess, was insignificant. These results and the data about much higher Cu concentration in the *A. yokoscense* root cell cytoplasm as compared with the concentration characteristic of other ferns allowed the authors to conclude that, in addition to the increased CEC of their root cell walls, some specialized intracellular mechanisms of Cu detoxification were responsible for a high *A. yokoscense* tolerance to Cu.

In studies of specific features of adaptation of one more highly tolerant Japan fern *Lygoduim japonicum* (Konno et al. 2005), substantial changes in matrix polysaccharides of the cell walls in fern prothallium were detected. It turned out that 20-day-long growth on medium containing 0.4 mM $CuSO_4$ resulted in the accumulation of 17.2 μmol Cu/g DW of the cell wall preparation. Cu excess in medium induced strong changes in the content and fraction composition of the basic cell wall components. The content of solubilized pectin was reduced by 53%, solubilized hemicellulose, by 82% of control, and the content of uronic acid increased markedly. With the usage of *endo*-pectate-lyase, it was also established that most of the Cu accumulated into the *L. japonicum* prothallium was tightly bound to galacturonic acids of homogalactouronan of the cell wall pectin.

In laboratory experiments performed on the aquatic plant *Potamogeton natans* (pondweed), the inhabitant of eutrophic lakes with increased HM concentrations, a distribution of Cu (and also Cd, Pb, and Zn) was studied (Fritioff and Greger 2006). The highest content of Cu was always found in the roots. The analysis of cell walls isolated from leaves and stems showed that 40–42% of Cu in leaves and 33–51% in stems were bound to the cell walls. The authors noted that CEC for Cd ($48 \pm 6$ and $51 \pm 5$ meqv $100\ g^{-1} \pm$ se for leaves and stems, respectively) was not correlated with the organic matter content in these plant organs, as distinct from usual state of affairs. The data for Cu are absent from this work.

A comparison of plant species differing in their tolerance to Cu in relation to the possible role of their cell walls was performed by Wei et al. (2008). *Sorghum sudanense* plants more tolerant to Cu in medium and less tolerant *Chrysanthemum coronarium* plants were grown in hydroponic culture in the presence of Cu excess (up to 50 μM). It turned out that, in *C. coronarium* shoots and their cell walls, total

Cu concentrations were much higher than in *S. sudanense*. However, in *C. coronarium*, the percentage of cell wall bound Cu was much lower (and the percentage of water-soluble Cu was higher). In contrast, in *S. sudanense*, substantially greater Cu amounts remained in the roots, and the percentage of cell wall bound Cu was also higher (76% of total content in the cell walls vs. 60% in *C. coronarium*). This was correlated with a strong increase in the content of uronic acids in *S. sudanense* induced by Cu excess and with their higher concentration as compared to *C. coronarium* (85 μg/g FW against 55 μg/g). The authors concluded that all these peculiarities could provide for a higher *S. sudanense* tolerance. Among the reasons for improved *S. sudanense* tolerance, it was also indicated that the 2.5-fold lower Cu concentration was present in this plant shoots, especially taking into account that more than its 15% (vs. 11% in *C. coronarium*) was retained by the cell walls. As a result, Cu load on *S. sudanense* protoplast turned out to be much lower than in *C. coronarium*.

Using cell walls isolated from *O. sativa* roots (including those inoculated with AMF *G. mosseae*), Zhang et al. (2009) demonstrated that Cu absorption was rapid, with saturation of binding sites being achieved within 10 min. The concentration dependence of Cu binding in isolated cell walls showed its high capacity and saturation at around 1.5 mM Cu. Along with significantly increased value of Cu-binding capacity of cell walls isolated from AMF-inoculated roots in comparison with noninoculated roots, a substantial difference was also observed during successive cell wall fractionation. It was shown that a significantly higher (approximately by 15%) Cu binding capacity (CEC equivalent) of the cell walls isolated from inoculated roots was largely determined by the increased contribution of the pectin-containing fraction into this characteristic.

Ke et al. (2007) compared two natural populations of *Daucus carota*, grown in soil culture from seeds collected from Cu contaminated site (CS) and uncontaminated site (UCS). In the presence of high Cu concentration (>400 mg/kg of soil), biomass accumulation in UCS population was reduced to 68–60% of control, but growth of CS population plants was not affected. Total and cell wall Cu contents were increased similarly (by 4.5 times) in leaves and roots of both plant populations. In the leaves, where Cu concentration was higher than in the roots, 51–54% of the whole leaf Cu was localized in the cell wall in both CS and UCS plants (vs. only 42–43% in control plants). Thus, any reliable contribution of extracellular Cu retention to the improved tolerance of CS plants was not established. In contrast, the higher level of Cu localization in vacuoles of CS population plants (14.2 vs. 9.1% in UCS plants) indicated the probability of intracellular mechanism of Cu detoxification functioning in *D. carota* plants.

Thus, all available approaches indicated that Cu was always detected in the apoplast, especially at its excess in environment. It is believed that the volume of the apoplast comprises only about 5% of total volume of plant tissues, but it represents a zone of a direct contact between the root and external medium (soil, for example), which could contain a great Cu excess in contaminated areas. Keeping in mind that the apoplast manifests weak metabolic activity, it might be expected that just the

apoplast could fulfill some protective functions against increased Cu concentrations toxic for plants. The materials presented confirm these expectations to some degree.

However, two circumstances interfere with the reliable assessment of the apoplast role in Cu detoxification. Firstly, appropriate methods for separation of apoplastic and symplastic Cu pools in intact organs (roots primarily) are absent. Therefore, reported basic indices, such as, for example, time of saturation, are very variable (from 4–6 h to 3 days); the data concerning a concentration dependence of apoplast pool filling are scarce, etc. As to the results obtained on isolated cell walls, there are great doubts relative to their equivalence to the initial cell wall state. Indeed, isolation of the cell polymeric matrix resulted frequently in the disturbance of its architecture and changes in chemical composition. Thus, lipid-free cell wall preparations were used in some experiments (Shi et al. 2008), ionically and tightly bound proteins and structural proteins were removed in other ones (Konno et al. 2005). However, direct data are available about the involvement of cell wall proteins in Cu immobilization. Neumann et al. (1995) demonstrated in studies of *A. maritima* EELS spectra that Cu binding to the cell walls is largely determined by the production of copper–protein complexes. The two copper-binding proteins were extracted from the cell walls. Remember also information concerning AMF-inoculated roots, e.g., a sharp increase in the CEC related to the presence of specific proteins in fungal hyphae. One of such protein is evidently a recently discovered glomalin. The confirmation of this result could open new possibility for plant genetic modifications.

Summing the material presented in this division, it may be concluded that, in spite of described difficulties, it is undoubtedly that the apoplastic Cu pool comprises a very substantial, sometimes dominating part of metal absorbed by the plant, especially at its excess in environment. Thus, the role of extracellular Cu pool must be taken into account when analyzing the problem of Cu detoxification.

## 8.4 Initial Steps of Intracellular Copper Detoxification

As was mentioned earlier, essentially all Cu ions are present in the cytosol in the bound form (Changela et al. 2003; Krämer et al. 2007). Thus, after Cu ions transfer across the plasma membrane with the help of membrane transporters, they are immediately subjected by chelating. The basic components of the defense system in the cytosol are S-containing ligands: phytochelatins (PC) and metallothioneins (MT). MT belong to the group of cysteine-rich proteins (8–10 kDa) encoded by the family of nuclear genes (Murphy and Taiz 1995; Cobbett and Goldsbrough 2002). As distinct from MT, PCs are oligomers of glutathione produced by the enzyme phytochelatin synthase (PCS) (Cobbett and Goldsbrough 2002).

Information concerning the role of MT and PC in detoxification of Cu excess is contradictory and sometimes mutually exclusive (Schäfer et al. 1997; Gonzalez-Mendoza et al. 2007; Guo et al. 2008).

We studied the involvement of MT and PC in Cu detoxification in *Brassica napus* L. plants grown in the presence of 10, 50, and 150 μM $CuSO_4$ for 10 days;

control plants were kept in the presence of 0.25 μM $CuSO_4$. In these experiments, we compared the time course of Cu accumulation in the intracellular Cu pool in the roots and leaves with the level of transcripts of *PCS* gene and two MT-encoding genes, *MT1* and *MT2*. Apoplastic Cu desorption was performed as described earlier (Ivanova et al. 2010; Kulikova et al. 2011); Cu concentration was measured with the atomic absorption spectrophotometer. Transcript content was assessed by routine methods (Kholodova et al. 2011). Plant material was fixed for analyses in 3, 6, and 24 h and also in 5 and 10 days of Cu treatment.

Figures 8.1 and 8.2 compare directly the Cu content in the intracellular (symplast) pool of the roots (Fig. 8.1) or its total content in the leaves (Fig. 8.2)

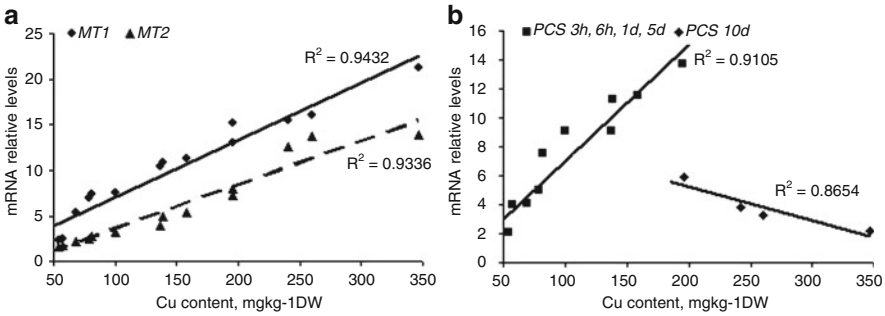


**Fig. 8.1** Correlation between Cu content in the root symplast and the level of *MT1*, *MT2* (**a**) and *PCS* (**b**) mRNAs in *Brassica napus* plants. Plants were kept on the 10, 50, and 150 μM $CuSO_4$ solutions from 3 h to 10 days. For estimation of Cu content in the roots, they were firstly washed with a large volume of running water, then with 10 mM EDTA for 15 min with stirring, and at last with distilled water. Cu content was determined using an AAS Labist-400 (Labist, Russia). mRNA relative level is the ratio of each gene mRNA amount in experimental rapeseed plants to the same mRNA amount in control plants (grown on standard nutrient medium). 18S rRNA was used as an internal standard. Measurements were performed in triplicate

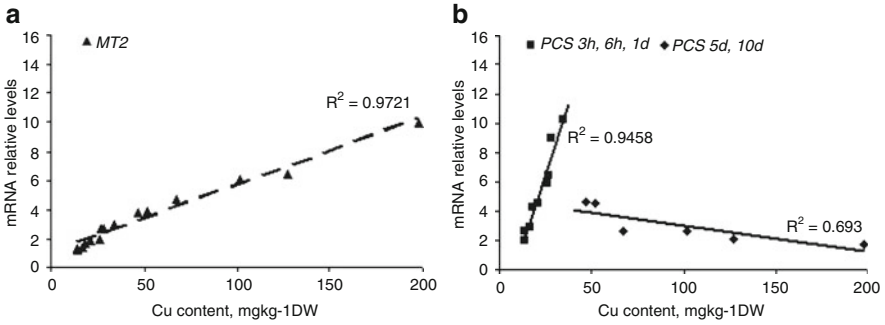


**Fig. 8.2** Correlation between Cu content in the *Brassica napus* leaves and the level of *MT2* (A) and *PCS* (B) mRNAs. Plants were kept on the 10, 50, and 150 μM $CuSO_4$ solutions from 3 h to 10 days. Cu content was determined using an AAS Labist-400 (Labist, Russia). mRNA relative level is the ratio of each gene mRNA amount in experimental rapeseed plants to the same mRNA amount in control plants (grown on standard nutrient medium). 18S rRNA was used as an internal standard. Measurements were performed in triplicate

with the level of mRNAs of tested genes at all used $CuSO_4$ concentrations in medium.

Under these conditions, Cu accumulation in the root symplast occurred very actively. For the first 3 and 6 h, Cu accumulated to the level of 68.7 and 99.6 μg/g DW, respectively. It continued to grow during experiment, attaining the highest concentration of 347 μg/g DW; this value exceeded initial Cu concentration (39.7 μg/g DW) by 8.7 times.

Already from the beginning of Cu treatment, the amount of *MT1* gene transcripts increased by 21 times as compared to the initial value (Fig. 8.1a). Activation of *MT2* gene transcription was less profound; nevertheless, the level of its mRNA increased until the end of experiment. It was unexpected that Cu excess in the root symplast initiated transcription of *PCS* gene, which product catalyzes the synthesis of phytochelatins, i.e., S-containing peptides of another group of ligands (Fig. 8.1b). During initial intracellular Cu accumulation, the extent of *PCS* gene activation was comparable with that of *MT1* gene, which is root specific (Guo et al. 2008). The highest value in the *PCS* transcripts was 13.7-fold higher that its initial level at the Cu content in the root symplast of 159 μg/g DW. However, further Cu accumulation was accompanied by a strong decrease in the level of *PCS* mRNAs.

Under similar conditions, in the presence of 10–150 μM $CuSO_4$, the leaves accumulated Cu noticeably slower than the roots. In 3 h, the initial Cu level in leaves (12.1 μg/g DW) was exceeded only by 1.1–2.2 times; however, by the end of experiment, the leaves accumulated up to 198 μg Cu/g DW, i.e., 16-fold more than its initial level.

As distinct from the roots, expression of *MT1* gene was not detected in leaves, confirming its root specificity. A statistically significant increase in the level of *MT2* transcripts was observed only in 24 h of treatment, the highest level, exceeding control values by 6.1–9.9 times, was attained at Cu concentration in leaves of 127–198 μg/g DW to the end of the experiment. In contrast to this slow response of *MT2* gene transcription to the Cu excess in the leaves, the *PCS* gene transcription was the earliest response: the level of *PCS* transcripts exceeded the initial level by 2–4 times at the less than twice increased Cu concentration. However, like in the roots, the level of *PCS* mRNAs reduced markedly with continued Cu accumulation on the background of a stable increase in the *MT2* transcript level (Fig. 8.2).

The correlation analysis was performed for tested indices. The analysis of the bulk of information obtained for roots showed that the coefficients of correlation between Cu concentration ([Cu]) and the level of mRNA were equal to +0.971 for *MT1* and +0.966 for *MT2* gene, for leaves, $r = +0.986$ for *MT2* gene. These data make very probable that the transcription levels of these genes were tightly connected with Cu concentration in the symplast of corresponding organs. In spite of the complex type of dependence between [Cu] and the level of *PCS* mRNA, the coefficients of correlation for each of the branches of this dependence were rather high: $r = +0.957$ and $r = -0.930$ for roots and $r = +0.972$ and $r = -0.832$ for leaves. It is unquestionable that the role of PCS in Cu detoxification must be studied in more detail.

Thus, as judged from the levels of tested gene transcription, in the roots, where Cu ion absorption by the cells was very intense, both groups of main S-containing ligands were evidently involved in the early steps of Cu detoxification. The products of two genes encoding MT could directly participate in this process. Activation of *PCS* gene expression could activate phytochelatin synthesis via PCS enzyme accumulation. As to the leaves, chelating Cu excess in them is seemingly organized rather rational. Indeed, since Cu penetration into the leaves was somewhat delayed, its early small excess was not evidently sufficient for *MT2* gene activation. In contrast, it was quite sufficient for very active transcription of the *PCS* gene. It is characteristic that *PCS* transcripts accumulated in the leaves even more actively than in the roots experiencing the large-scale Cu penetration into the cells. When the Cu content in leaves attained 70 μg/g DW, the relative levels of *PCS* and *MT2* mRNAs became equal, and then the level of *MT2* mRNAs exceeded that of *PCS* mRNAs. Such behavior of two ligand groups at excessive Cu concentrations in the rapeseed leaves could be considered as their coordinated functioning in Cu excess detoxification, which was manifested at the earliest step of Cu action on rapeseed plants. Similar interaction between phytochelatins and metallothioneins in Cu detoxification, realized under different conditions, has been earlier observed in black mangrove *Avicennia germinans* (Gonzalez-Mendoza et al. 2007) and Arabidopsis (Guo et al. 2008) plants.

## 8.5 Nanoparticles of Metallic Copper in Plants

Recent years are marked by an intense development of nanotechnologies, which start to be used not only in diverse fields of industry but also in medicine and plant studies. In this connection, the interaction between nanoparticles (NPs) and plants was focused in recent studies (Nowack and Bucheli 2007; Ma et al. 2010). Below, we consider briefly two aspects related closely to the issue.

### *8.5.1 Production of Copper Nanoparticles by Plants*

A possibility of the presence within the plant cell of metallic Cu usually entering plants, like other metals, as ions does not seem impossible or exotic. $Cu^{2+}$ is reduced to $Cu^{+}$ in soil in the near-root environment, and compounds comprising $Cu^{+}$ were detected in plant roots (Naftel et al. 2007). But most important is that plant cells comprise some oxido-reductively labile metabolites capable of metal ion reduction to the metallic forms. Among them, ascorbic acid evidently plays a crucial role as a mild reductant (Manceau et al. 2008).

In recent years, it was established that the extracts from plant tissues could be used for reduction of some metal ions. It is especially interesting that, as a result of this reduction, metals produce nanoparticles (NPs) because the sizes of individual

structures produced are less than 100 nm in more than one dimension (which is characteristic for nanostructures) (Nowack and Bucheli 2007).

For obtaining metallic Ag NPs from $AgNO_3$, Jha et al. (2009) used 50% ethanolic tissue extracts of various ecological plant groups: xerophytes *Bryophyllum* sp., mesophytes *Cyperus* sp., and hydrophytes *Hydrilla* sp. With the usage of X-ray diffraction (XRD) spectra technique, it was detected the formation of cubic Ag NPs. With the usage of TEM micrographs, the sizes of particles were found to be in the range of 2–5 nm. The authors believe that soluble flavones, quinines in plant tissue extracts were involved in the reduction of silver ions at 40°C, which lasted for several hours.

Haverkamp and Marshall (2009) studied the mechanism of metallic NP formation in plants as exemplified by several Ag salts absorbed by *Brassica juncea*. The upper limit for reduced Ag metal NPs was established as 0.35% Ag of plant dry weight, and it depended on the reducing capacity of the plant under experimental conditions. It became clear why, along with Ag, only Au and Cu NPs with the reduction potential of at least 0.04 V were detected in plants. From a review of the electrochemical potential observed, it was proposed that metal NP formation in plants is restricted to those elements, whose salts have a potential for reduction to metal above about 0 V. This indicates that the plant metal NP production will be limited to the precious and semiprecious metals. Along with already mentioned Ag (up to 0.80 V), Au (1.0 V), and Cu (0.35 V), Pd (0.64 V) and Pt (0.74 V) belong to this group.

It should be noted that, in recent years, the interest to the production of NPs with the help of plants increased markedly, in particular in connection with their usage in medicine. Indeed, the biological formation provides for the production of low-cost, energy-efficient, and nontoxic metallic NPs (Thakkar et al. 2010). A possibility exists of NP usage for plant life control, including for nanogenetic crop manipulations (Nair et al. 2010).

### 8.5.2 *Action of $Cu^0$ Nanoparticles on Plants*

As is seen from the material presented in the previous division (Sect. 8.5.1), metal ions, Cu in particular, may be reduced to the metallic form in the living plant (Haverkamp and Marshall 2009), although the extent of this process is rather limited. In contrast, natural and engineered sources of NPs are rather sizeable; among the natural sources, volcanoes are of importance. As to the production of engineered nanoparticles (ENPs), according to the evidence from The Royal Society and Royal Academy of Engineering 2004, presented by Navarro et al. (2008), in 2004 the annual global production of ENPs was of the order of $10^3$ tons and it is expected to increase to $10^4$–$10^5$ tons per year after 2010. In this connection, the effects of ENPs on plants and the role of the cell walls in defense against a potential NP threat should be closely considered.

The cell wall is a barrier on the route of ENP entry into the plant cell. The diameter of pores across the cell wall is within the range of 5–20 nm, and this

determines its sieving properties (Fleischer et al. 1999). Such sizes of pores would limit penetration of large NPs. At the same time, it is known that newly synthesized cell walls manifest the higher permeability; in addition ENPs themselves might induce the formation of new, bigger than usual pores. Like for macromolecules, endocytosis can serve as a main way for translocation across the plasma membrane (Ovecka et al. 2005). Thus, there are no principal obstacles for NP penetration into and spreading over the plant. In this connection the question arises as to the nature of NP and especially ENP action on plants and a possible role of the cell wall in this interaction. Below, we present scarce available data concerning Cu ENPs.

When studying ENP action on germination of lettuce seeds, Shah and Belozerova (2009) introduced Cu in soil as copper nanosize activated powder to a final concentration of 0.013 or 0.066%. The shoot/root ratio was used as an index of possible NP action. In 11 days at the highest Cu concentration, this ratio increased significantly from 1.92 to 2.70, which evidently reflected the stronger inhibitory effect on root than shoot growth. This effect was manifested only after a 15-day-long soil pretreatment with NPs; the authors believe that this fact indicates a possibility of indirect Cu NP action on lettuce seedlings.

In more thorough investigations performed by Stampoulis et al. (2009) on *Cucurbita pepo*, the effects of Cu ENPs and bulk Cu powder were compared. Any effect on germination was not found. However, 15-day-long growth in hydroponic solution revealed the stronger inhibitory action of Cu ENPs on root length and biomass as compared with bulk Cu powder. Thus, root length was reduced by 77% in treatment with Cu ENPs, but only by 64% in treatment with bulk Cu powder. A decrease in biomass attained 90% in treatment with Cu ENPs, but only 69% in treatment with bulk Cu powder. An additional verification showed that a possible ionization of a small part of Cu during experiment did not change substantially a conclusion about the higher phytotoxicity of Cu ENPs as compared with that of bulk Cu powder.

Similar conclusion was made by Lee et al. (2008), who used *Phaseolus radiatus* and *Triticum aestivum* seed germination as a toxicity test for Cu ENPs. The seeds were germinated in Petri dishes on dual agar media for homogeneous exposure of plant roots to NPs. In *P. radiatus*, 50% growth inhibition of seedlings was observed at the Cu NP median effective concentration of 335 mg/l, whereas in *T. aestivum*, at 570 mg/l. It was also checked that the concentration of copper ions released during sonication process was very low and the apparent toxicity clearly resulted from Cu NPs. For a comparison of various plant species, a bioaccumulation factor, [Cu] in plants (mg/kg dry tissue)/[Cu] in media (mg/l), was used. Its values were 8 and 32 l/kg for *P. radiata* and *T. aestivim*, respectively, which reflected, in particular, specific root morphology of these plant species. The application of TEM for monitoring Cu localization showed the presence of individual and aggregated NPs in the cell walls and inside of the root cells of *P. radiata* and *T. aestivum*. Bigger deposits were found at the higher concentrations of Cu NPs. Using TEM-EDS and EDS-scanning technique, the high Cu concentration in deposits was demonstrated, although only a small portion of NPs could be transported within the plant from roots to shoots.

In the field experiments, the common wetland plants, *Phragmites australis* and *Iris pseudoacorus,* were grown in $Cu^{2+}$-contaminated soil (Manceau et al. 2008). Using synchrotron microanalysis, Cu grains 5–20 μm in size were observed in the rhizosphere of *P. australis* on the root surface in the sites of its contact with the fungal mycelium and in the cortical parenchyma, but not in the central vascular cylinder of the root. In *I. pseudoacorus,* Cu spots were detected by XRD in the zone of root hairs and mycorrhizal hyphae and, using EXAF spectroscopy, were identified as $Cu^0$. X-ray diffraction confirmed that Cu grains were the aggregates of NPs; some of them were represented by individual NPs. The size of $Cu^0$ NPs was evidently within the range of 1.0–15.0 nm. The authors concluded that copper was reduced biotically, evidently with the involvement of organic molecules as templates to control the shape and size of metallic nanoparticles. They believe that this newly identified mode of copper biomineralization by plant roots (and mycorrhizal hyphae) under copper stress would prevent copper from entering the food chain.

Thus, $Cu^0$ NPs in plants could arise in the process of $Cu^{2+}$ or $Cu^+$ reduction within the plants or penetrate from environment. In spite of the scarcity of experimental data, an increased toxicity of $Cu^0$ ENPs (in comparison with Cu ions) seems rather likely. In the connection with predicted global production of ENPs, the expanded and deepened analysis of their action on plants is required.

## 8.6 Conclusion

The analysis of presented data concerning the mechanisms of copper detoxification and the maintenance of its homeostasis in plant cells at its excessive content in environment allows some generalizations.

It should be first noted that a possibility of metallic copper ($Cu^0$) presence in the plant was until now essentially outside researcher attention. In recent years, it became evident that $Cu^{2+}$ reduction to $Cu^0$ in the plant cells is not only allowable theoretically but, in some cases, experimentally proven, e.g., metallic copper is detected in plant cells. It is also established that $Cu^0$ particles have nanosizes. It is of especial importance that copper nanoparticles were observed not only in the apoplast but also in the cytoplasm. A rather limited material obtained in this field did not allow unambiguous answer to the question whether the formation of copper nanoparticles is a novel way of excess Cu detoxification or, in contrast, these particles represent a real threat to plant life. It is undoubtedly that this field of research will develop violently in the nearest years.

The second important aspect of the problem of Cu detoxification and the maintenance of its homeostasis is an essential absence of free Cu ions in the plant cell cytoplasm (Changela et al. 2003). This is a consequence of the functioning in plants of very efficient systems of its chelating and compartmentation. Currently, a great progress is achieved in studying Cu detoxification mechanisms operating within the cells (in symplast). Indeed, well-grounded experimental publications

and comprehensive reviews destined to this problem appear regularly. Our experiments showed that, at early steps of Cu excess detoxification in the plant cell cytosol, both metallothioneins and phytochelatins evidently play an important role. A good coordination between functioning of these two groups of Cu high-molecular ligands was also demonstrated.

Of considerable value is also the notion that, along symplastic, its apoplastic pool, which is characteristic only for plants, is also involved in Cu detoxification. These two pools differ fundamentally in the mechanisms used for the restriction of Cu ion bioavailability, which primarily follows from differences in the types of Cu chemical bonds dominating in these two compartments. In recent years, due to the development of new methods for Cu quantification in various cell compartments, including quantitative microscopy, vast information is accumulated concerning the apoplast participation in Cu immobilization, which allows a discussion of a possible role of this phenomenon in Cu detoxification.

The role of the apoplast in Cu excess detoxification attracted the attention of researchers since the 1970s. The idea was put forward that HM association with plant cell walls is a primary mechanism of tolerance because Cu accumulation in the cell wall hinders its penetration into more sensitive cell metabolic sites in the root. Despite the alternative view is known (Ernst et al. 1992), the materials presented earlier show that, in some plants, up to 60–92% of total copper of the root cells was localized in the apoplast. This shows a necessity of further intense investigations of the cell wall role in Cu retention.

It should be noted that even under ordinary Cu content in medium, cell walls immobilize a great amount of Cu (up to 43–47% of total Cu content in leaves and roots). Plant growth in the presence of Cu excess results not only in the increased proportion of Cu in the cell walls but also in the substantial increase (up to 100-fold) in the Cu concentration (Nishizono et al. 1987). The important conclusion is that usually a potential capacity of cell walls relative to Cu absorption is used only insignificantly. It is also worth mentioning that a comparison of two plant species differing in their tolerance to Cu showed that Cu retention by the cell walls in the roots and leaves of more tolerant species was higher that in less tolerant species.

In all likelihood, improved tolerance due to enhanced Cu immobilization in the apoplast could be realized at the initial stage of growing root penetration into Cu-enriched soil. This response disappears later, which could indicate a gradual transfer of Cu, initially held by the cell wall, inside the cell.

It is not excluded that plant growth in Cu-enriched medium could initiate an increase in the cell wall CEC. This is indicated by some researchers detecting changes in the composition and structure of the cell walls as a result of long-term action of Cu excess. In particular, it was established that Cu induced an increase in the contents of pectin and hemicelluloses in the cell walls, changes in their fraction composition, accumulation of uronic acids, which occurred only in species tolerant to Cu, and also an increased content of lignin and suberin (Llugany et al. 2003; Konno et al. 2005; McKenna et al. 2010).

At the same time, in some cases an improved plant tolerance to excess Cu in medium may be provided otherwise than by the expansion of its apoplastic pool.

Thus, at the evaluation of the ways of Cu detoxification under natural conditions, the contribution of AMF was clearly undervalued. An increased Cu tolerance of the plants colonized by AMF is explained by the effects of Cu entrapping or filtration. This important positive AMF effect is evidently determined by the much higher cell wall CEC values characteristic of fungal hyphae in comparison with plant cell walls. Accepting the urgency of the problem of purification of HM-contaminated territories and keeping in mind the absence of plants promising for phytoremediation of Cu, more attention should be paid to cultivation of AMF-colonized plants on Cu-enriched soils.

Thus, the apoplastic pool of copper includes a great, sometimes dominating part of Cu absorbed by the plant, especially at its excess in environment. Chemical and structural changes occurring in the cell wall under the influence of Cu excess (and also under the influence of AMF) allow consideration of the cell wall as a substantial component in the system of plant adaptation to HM action.

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