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# COPPER - INDUCED CUPRIC- AND FERRIC-CHELATE REDUCTION BY INTACT BARLEY ROOTS

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**Summary**. The present study reveals the relationship between copper uptake and cupric-chelate reductase activity alteration as well as the copper effects on ferric-chelate reduction and HCF III reduction by intact young barley plants, grown under conditions of calcium nutrition. After 24 h copper uptake from solutions containing different micromolar cation concentrations, cupric-chelate reductase activity increases proportionally to the copper treatment and depends on the type of copper complexing agent. Our results are in support of the enhancement of a standard redox-system activity, operating at root cells plasma membrane, thus facilitating the reduction of cupric-chelates, some ferric-chelates, and HCF III to a different extent. Another proof of standard redox-activity stimulation is based on the results with its well-known effector – Vit.$K_3$ or menadione as an accelerator of electron transport through PM. Our results show high increase of Cu(II)-His RA and Fe(III)-HEDTA RA after pretreatment of barley roots with menadione. The strong additive effect of copper ions to Vit.$K_3$ action also sustains the assumption for activation of the standard or constitutive redox-system involved in cupric- and ferric-reduction by barley roots.

***Key words***: copper uptake, calcium nutrition, barley roots, standard redox-system, cupric-chelate reductase activity, ferric-chelate reductase activity, hexacyanoferrate(III) reduction, redox-effectors.

***Abbreviations***: SD – standard deviation, FW – fresh weight, HCF III RA – hexacyanoferrate(III) reductase activity, SRS – standard redox-system, PM – plasma membrane, Phen – 1,10-phenantroline monohydrate, His – histidine,

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HEDTA – hydroxy ethyl-ethylenediamine-triacetic acid, Dicumarol – 3,3-methylene-bis(4-hydroxycoumarin), menadione or Vitamin $K_3$ – 2-methyl-1,4-naphthoquinon, DMSO – dimethylsulphoxide.

## Introduction

Plasma membrane of plant root cells contains a redox-system consisting of redox-components and enzymes capable of conducting electrons across the plasmalemma. Electron donators include some cytosolic reductants providing charges to various apoplastic acceptors or external oxidants such as ferric-chelates, ferricyanide or cupric-chelates for their reduction (Rubinstein and Luster, 1993; Holden et al., 1995; Döring et al., 1998). Under conditions of adequate iron supply, this so-called "standard" or constitutive redox-system, associated with PM of root cells might regulate ion transport, membrane energization, proton flux (Bienfait and Lüttge, 1988). The precise role and other functions of SRS, concerning its involvement into mechanisms of cation uptake, however, are not well understood, (Döring et al., 1998). The most clear function of redox-system is connected with the iron acquisition by plants (Guerinot and Yi, 1994; Moog and Brüggemann, 1994). Uptake of iron by all dicots and nongrass monocot plants requires an obligatory step of reduction of $Fe^{3+}$ to ferous ion performed by PM- associated ferric-chelate reductase (Römheld, 1987; Bienfait and Lüttge, 1988; Bagnaresi and Pupilo, 1995; Connolly and Guerinot, 1998). Reduced iron moves through PM of these plants by help of specific for bivalent ions transporter (Römheld, 1987; Eide et al., 1996). The mechanisms of copper uptake by plant roots, however, are not well understood and are very fragmentary studied. The reactivity of copper ions to form stable bivalent complexes offers the possibility of their participation in redox-reactions at the plasma membrane of plant cells as was shown for yeast cells. Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae* was shown (Hasset and Kosman, 1995) and a high-affinity, strictly energy-dependent copper uptake mechanism was found (Dancis et al., 1994a). The regulation of copper and iron uptake by yeast cells includes some common mechanisms and close relations (Dancis et al., 1994b; Stearman et al., 1996). Recently we have described the capability of intact roots of pea and maize plants to reduce cupric-chelates specifically, independently of Fe nutrition (Babalakova and Schmidt, 1996; Babalakova et al., 1998). In vivo treatment with copper ions in solutions additionally increase the reductase activity. The reduction of different cupric-chelates by root plasma membrane vesicles was demonstrated and redox-proteins with high cupric- and ferric-chelate reductase activity in purified plasma membranes were isolated and partially purified (Babalakova et al., 1998; Babalakova et al., 2000 a and b). The precise involvement of ion-reduction process in the mechanisms of copper uptake through the plant root plasmalemma is not yet known. (Holden et al., 1995; Babalakova and Schmidt, 1996). Scare data describe the suggestions of some authors

that copper deficiency like iron deficiency could stimulate ferric-chelate reductase activity in pea root cells and this enzyme is also capable to reduce cupric-chelates (Welch et all., 1993, Cohen et all., 1997). Other results have brought to the conclusion that copper reduction is fulfiled only in the presence of free copper ions in excess, again for dicots plants (Holden et all., 1995). In our earlier investigations we have discussed the presence of an active, metabolic-dependent component in assimilation of copper ions by pea roots, using different inhibitors and ionophores ( Babalakova et all., 1988). Some data supporting an active mechanism for copper uptake by plant tissues have been reported (Veltrup, 1976; Veltrup, 1977; Bowen, 1981). The direct relation between ion-reduction by plant roots and the uptake processes is still not clear. In the present study we have compared the rate of uptake of copper ions and the induction of cupric- and ferric-chelate reductase activity in roots of intact barley plants, grown in conditions of calcium nutrition and young enough to sustain adequate plant iron content. The results of our study are in support of some correlation between the enhancement of cupric-chelate reductase activity and the uptake of micromolar concentrations of copper ions by intact barley roots. Our data demonstrate that standard or constitutive redox-system operating at plasma membrane of root cells facilitates the reduction of cupric- and ferric-chelates to a different extent depending on the type of complexing agents.

## Materials and Methods

### Plant material

Experiments were carried out with 8-9-day old barley plants *Hordeum vulgare* L., cv. Kakaihadaka, Japan), grown in an environmental room as hydroponic culture on solutions containing 0.1 mM $CaCl_2$, under controllable light conditions – 160 µmol.$m^{-2}$.$s^{-1}$ proton flux density and at a day/night temperature of 25° C/22° C. *In vivo* treatment with copper ions was provided for 6-, 12-, and 24 hours with 10, 20 and 30 µM copper chloride in solutions containing 0.1 mM $Ca^{2+}$, pH 5.5.

### Determination of total uptake of copper ions by plant roots

The uptake of $Cu^{2+}$ by intact plant roots from solutions was evaluated by the changes of the corresponding cation concentrations determined from the beginning to the end of experiments for different periods of time by means of AAS “Perkin Elmer”, model 3030 and was expressed as µg Cu per g fresh weight of roots per h. (Babalakova et al., 1988). Control plants were grown in solutions containing calcium chloride. Copper ion absorption from solutions with different concentrations was estimated as percentage to the initial ion concentration in the solutions and was compared for various periods of time.

### Enzyme activity essay

Cupric chelate reductase activity in root cells of intact barley plants was measured before and after the *in vivo* treatment with copper ions as previously described (Babalakova and Schmidt, 1996; Babalakova et al., 1998) using different cupric- complexes as electron acceptors like Cu(II)-Citrate, Cu(II)-Phen, Cu(II)-His and Cu(II)-HEDTA, prepared at different ratios of Cu to substances (1:1 for HEDTA, 1:2 for Phen and His, and 1:3 for Citrate) as Tris-KOH salts, pH 6.5. Ferric-chelate reduction was performed using Fe(III)-HEDTA complex and Fe(III)-Citrate (for comparison with cupric chelates) according to the methods described by Babalakova and Schmidt,1996; Alkantara et al.,1994), using the protocol of Blair and Diehl (1961). Hexacyanoferrate III (HCF III) was also used as a common impermeable exogenous electron acceptor for measuring the activity of standard or constitutive redox-system at plasma membrane of root or other cells (Valenti et al.,1991) and for comparison with other ferric- and cupric-chelates. The reductase activity was expressed in nmoles reduced substrate per g of root FW per h.

### Redox-inhibitors and effectors action

The effects of redox-effectors Dicumarol and menadione (vitamin $K_3$) on the reductase activity in barley roots were performed with 1 h pretreatment of the roots with 100 µM of each substance dissolved in DMSO and buffer (Döring et al.,1992).

The experiments with intact plants were repeated at least 3 times with 6 to 8 replicates in each variant and the values were expressed with SD.

## Results and Discussion

Treatment of barley plants with copper ions applied at micromolar concentrations led to increased Cu uptake by root cells depending on the concentrations and duration of treatment (Table 1). Copper absorption showed one and the same extent of uptake – about 62 to 68% of the initial ion concentration in the medium (Fig. 1 – 6-h period

**Table 1.** Uptake of copper by intact barley roots depending on the copper concentration and uptake duration.

| Plant Variants | Uptake of copper in µg Cu.g$^{-1}$ FW | | |
|---|---|---|---|
| | 6 h uptake | 12 h uptake | 24 h uptake |
| Ca + 10 µM $Cu^{2+}$ | 30.7±1.6 | 50.0±2.1 | 50.5±2.5 |
| Ca + 20 µM $Cu^{2+}$ | 56.8±2.3 | 82.3±4.2 | 96.2±4.8 |
| Ca + 30 µM $Cu^{2+}$ | 76.3±4.1 | 120.7±6.7 | 126.1±6.8 |

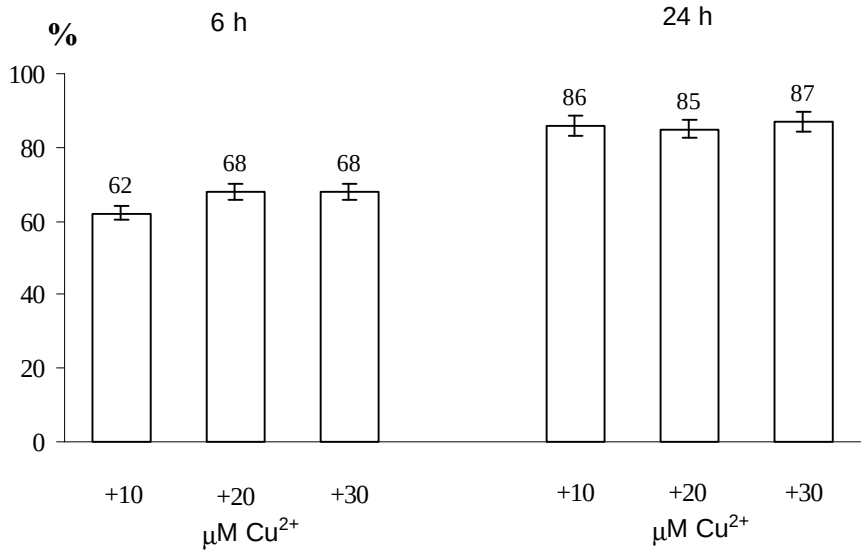


**Fig. 1**. Copper absorption by intact barley roots after 6- and 24-h period of uptake (in % to the initial ion concentration in the solution)

uptake) and about 85% for the uptake duration of 24 h providing the information for a good regulation of the uptake (Fig. 1). Pretreatment for 24 h with copper and after that removing the copper from the solutions showed one and the same rate of copper ions release (about 10 to 15% regardless of copper concentration – data not shown). These results demonstrated the specific regulation of uptake and copper release processes in barley roots. Despite the importance of copper as a micronutrient for plant growth, there have been relatively few studies of the mechanisms of copper uptake (Veltrup, 1976; Veltrup, 1978, Bowen, 1981, Babalakova et al., 1988,). All tracer flux measurements performed by different authors did not demonstrate a clear distinction between actual membrane flux through plasmalemma and cation binding outside cells. Using EDTA washing after pretreatment by copper ions we have previously demonstrated that about 90% of the copper content in solutions containing 10 μM $Cu^{2+}$ was uptaken by the roots of pea or maize plants (Babalakova, et al., 1988, Babalakova et al., 1998). An application of some metabolic inhibitors for measuring the copper uptake resulted in an inhibition of about 30 to 50% showing an active component of the uptake by pea plants (Babalakova et al., 1988). The close relation between uptake of copper and redox-system activity at PM has been demonstrated till now only for green alga *Chlamydomonas* (Hill et al., 1996). The root cells of young barley plants have shown capacity to reduce each of the cupric- and ferric-chelates applied as substrates. Barley plants belong to the strategy II plants without inducible "turbo"- reductase in conditions of iron defficiency (Römheld, 1987; Bienfait and Lüttge, 1988; Moog and Brüggemann, 1994).Under conditions of adequate iron supply for young

roots, however, the functions or precise role of so-called 'standard' or constitutive redox-system, associated with plasma membrane of root cells and capable of reducing different exogenous acceptors of electrons are not well defined (Babalakova and Schmidt, 1996). The cupric-chelate reductase activity of intact plant roots using different substrates is presented in table 2 and its stimulation by *in vivo* treatment with copper ions applied at concentrations of 10 and 20 µM is also shown. The extent of reductase activity increase after uptake of copper ions was different in the presence of Cu(II)-Citrate, Cu(II)-Phen and Cu(II)-HEDTA (about 45 to 90% stimulation at 10 µM Cu and from 62 to 170% – at 20 µM Cu). The reductase activity with Cu(II)-HEDTA as an acceptor of electrons was lowest but its percent activation after an *in vivo* short-term application of copper ions was highest (Table 2). Cupric(II)-Citrate

**Table 2**. Cupric-chelate reduction by intact barley roots (using different electron acceptors as substrates) after treatment with free copper ions, applied at µM concentrations for 24 h

| Variants | Reductase activity with different cupric-chelates in nmol Cu(I).$g^{-1}$ FW.$h^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| | Cu(II)-Citrate (1:3) | | Cu(II)-Phen (1:2) | | Cu(II)-HEDTA (1:1) | |
| | Value | % to control | Value | % to control | Value | % to control |
| Ca-control | 280±25 | 100 | 255±21 | 100 | 130±11 | 100 |
| + 10 µM $Cu^{2+}$ | 400±35 | 143 | 455±38 | 178 | 250±15 | 192.3 |
| + 20 µM $Cu^{2+}$ | 455±40 | 162.5 | 580±52 | 227.5 | 350±18 | 269.2 |

was thought to be the natural substrate for the plasma membrane redox-system of root cells and the induction of reductase activity after in vivo treatment by copper ions was found in barley roots. Due to various ratios of copper ions to ligands, the mid-point redox-potential for the three substrates might be different. Another explanation for the different rate of activation of the reductase activity after uptake of copper ions referred to the presence of several redox-proteins at the plasma membrane with different affinity towards substrates. We have described recently the association of different redox-proteins with purified plasma membranes isolated from maize roots (Babalakova et al., 2000-a,b). The capability of the redox system to provide electrons to each of the substrates was different and its capacity increased after *in vivo* copper ions application. The comparison with ferric-chelate reduction by PM redox-system supported the view for different redox-enzymes (table3). With HCF III as a common substrate for the demonstration of a standard reductase activity ( Böttger et al., 1995; Döring et al, 1992) we found similar acceleration of this activity after Cu-ions treatment. Ferric-chelate reductase activity measured with Fe(III)-Citrate as a substrate, however, has slightly altered under the influence of free copper ions in the solutions (Table 3). Ferric-citrate is also thought to be the natural substrate for the ferric-chelate reductase at the plasma membrane of root cells. The use of Fe(III)-HEDTA as an elec-

**Table 3.** Influence of *in vivo* copper ions treatment on the activity of ferric-chelate reductase and HCF(III) reductase at plasma membrane of intact barley roots.

| Variants | Reduction of Fe(III)-chelates in nmol Fe(II).$g^{-1}$ FW.$h^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| | Fe(III)-Citrate | | Fe(III)-HEDTA | | HCF(III), (FeCN) | |
| | Value | % to control | Value | % to control | Value | % to control |
| Ca-control | 580±25 | 100 | 325±28 | 100 | 975±75 | 100 |
| + 10 µM $Cu^{2+}$ | 590±26 | 101.7 | 235±25 | 72.3 | 1280±85 | 131.3 |
| + 20 µM $Cu^{2+}$ | 610±30 | 105.2 | 205±18 | 63.1 | 1450±85 | 148.8 |

tron acceptor of ferric-chelate reductase brought to some decrease of the redox activity in the presence of copper ions. It was found that under iron deficiency conditions free copper ions inhibited ferric-reductase activity at PM of dicot plants (Alcantara et al., 1994; Schmidt et al., 1997). We obtained different behaviour of both cupric- and ferric-reductase activities with the chelating agent HEDTA upon copper ion treatment. The explanation is in accordance with the opinion for different redox activities, engaged with cupric- and ferric-chelate reduction (Babalakova and Schmidt, 1996) and additionally, reductases in PM of monocot plants might possess different properties from the reductase engaged in ion reduction in dicot plants (Bienfait and Lüttge, 1988; Berczi and al., 1998). Besides the inhibition of Fe(III)-reduction by copper ions only with Fe(III)-HEDTA (between 27% and 37% depending on the copper ion concentrations), but not with Fe(III)-Citrate, reflected the various responses of membrane reductase towards different ferric-complexes (Babalakova and Schmidt, 1996; Schmidt, 1994). It is also important to underline that in our experiments copper ion treatment provoked the increase of standard-like reductase activity and this constitutive redox-activity working with cupric-chelates in conditions of adequate iron supply provided the trans-membrane transport of electrons, needed for cupric-reduction as an important process for monocot plants. Reduction of cupric- to cupro-ion was thought to facilitate the uptake of copper from strong cupri-complexes by increasing the mobility of reduced cation form, thus becoming more available for transport mechanism at plasma membrane of cells (Hasset and Kosman, 1995; Stearman et al., 1996). One support of our results showing the role of cupric-reduction by intact plant roots can be considered from the study of Bell et al. (1991) with intact maize plants. The authors used nutrient solution containing cupro-complexes as a copper source and obtained the similar copper content in shoots and the same yield as plants supplied with bivalent copper ions. Two suggestions could be drawn from these results: firstly plants could use cupro-complexes in the same manner as cupri-ions, and secondly, plant root cells might contain a mechanism for an oxido-reduction process of copper ions or complexes needed for the element acquisition based on the conclusion of the authors that Cu(II) and not Cu(I) controlled copper availability (Bell et al., 1991). Our results showed that the activation of cupric-chelate reductase activity

by *in vivo* application of $Cu^{2+}$ correlated with the uptake of $Cu^{2+}$ by barley roots. Another support of the opinion for the standard reductase activity acceleration after uptake of copper ions was based on the results with redox-effectors treatment of intact barley plants. The lipophilic agent vitamin $K_3$ (menadione) was used as a stimulator of a "standard" HCF III-reductase activity at plasma membrane of different cells (Barr et al., 1990, Döring et al., 1992). Dicumarol is thought to be a competitive inhibitor and antagonist of vit.$K_3$ action. Its highly hydrophobic molecule was supposed to enter the lipophilic part of the membrane and interacting with the electron-transporting chain it produced the inhibition of the electron transfer to HCF III (Döring et al., 1992). Cupric-chelate reductase activity of barley roots was activated after pre-treatment with menadione and cupric ions additionally highly accelerated the reductase activity (more than 500%, Table 4). This additive effect of copper ions and Vit.$K_3$ might suggest facilitated electron transfer from the cytoplasmic donors of electrons (NADH or NADPH) to the cupric-chelate acceptors outside root cells. Some evidences for the presence of quinones like K-type vitamins in the plant plasma membranes were presented and some suggestions were given for their participation in the electron-transporting chain of PM redox-system (Lüthje et al., 1998). The additive stimulating effects of menadione and copper ions on cupric-chelate reductase activity is reported for the first time. The exact mechanism of enzyme activity acceleration by Vit.$K_3$ rests to be resolved and needs further investigations. Fe(III)-chelate reductase activity was also stimulated by Vit.$K_3$ action to a smaller extent and some additive effects of copper ions was received (Table 4). Pre-treatment of intact barley roots with the antagonist dicumarol led to the inhibition of cupric-chelate reductase activity and could be explained by blocking of the electron transfer as was shown for the standard redox-system (Barr et al., 1990). We have used menadione effects and its antagonist towards redox-activity of barley root cells in order to explain the idea for a standard redox-activity increase under adequate iron supply and also, the

**Table 4**. Influence of redox-effectors (dicumarol and menadione) on the cupric- and ferric-chelate reductase activity in barley roots.

| Plant variants and treatments | Cu(II)-His RA in nmol.$g^{-1}$ FW.$h^{-1}$ | | Fe(III)-HEDTA RA in nmol.$g^{-1}$ FW.$h^{-1}$ | |
|---|---|---|---|---|
| | Value | % to control Ca | Value | % to control Ca |
| Ca-control | 245±22 | 100 | 325±31 | 100 |
| + menadione | 455±30 | 186 | 560±45 | 172 |
| + dicumarol | 132±10 | 54 | – | – |
| Ca + 10 µM $Cu^{2+}$ | 375±35 | 153 | 235±21 | 72 |
| + menadione | 1252±75 | 511 | 755±62 | 232 |
| + dicumarol | 162±14 | 43 | – | – |

induction of this activity by application of free copper ions. Although a cupric reductase activity has been measured in algae and plants (Jones and Morel, 1998; Welch et al., 1993; Holden et al., 1995; Babalakova and Schmidt, 1996; Cohen et al., 1997) it is not clear whether these activities are directly related to copper uptake. The results with *Chlamidomonas* devoted to the regulation of copper uptake in response to copper availability demonstrated, however, that cupric-reductase had to be a general component of a copper uptake pathway (Hill et al., 1996).

High cupric-chelate reductase activity of native purified membrane vesicles, isolated from maize roots supported the assumption of the importance of cupric-reductase in plant ion transport processes and the diversity of the redox-enzymes in plant root plasmalemma (Babalakova et al., 2000 a,b).

In conclusion, we can underline that short-term uptake of copper ions by barley plants leads to the enhancement of cupric-chelate reductase activity, associated with PM of intact root cells. The reduction capacity increase is mainly due to the stimulation of plasma membrane standard redox-system, responsible for cupric- and some ferric-chelates reduction to a different extent depending on the type of complexing agents. Intensive research is necessary in the future to elucidate the mechanisms of copper and iron interactions at a membrane level as well as the regulation of reduction processes at the PM and their relation to the transport of both ions in plant root cells.

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