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Reactions of root plasma membrane redox activities in iron-deficient cucumber plants after application of ionic and chelated copper

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ABSTRACT The effects of ionic ($CuSO_4$) and chelated forms of copper (Cu(II)HEDTA, where HEDTA is N-(2-hydroxyethyl) ethylenediamine triacetic acid, applied at micromolar concentrations in nutrient solutions of cucumber plants grown hydroponically under conditions of iron deficiency (–Fe), were studied. Changes of plasma membrane reductase activity (PMRA) of intact roots after treatment with ionic or chelated copper were followed in (+Fe) and (–Fe) cucumber plants. Iron deprivation in nutrient solution provoked a great increase of ferric-chelate reductase activity (with substrate of Fe(III)HEDTA) and accelerated the cupric-chelate reductase activity (measured with Cu(II)Citrate as an electron acceptor) as well as the hexacyanoferrate(III) [HCF(III)] reductase activity. Continuous application of cupric ions in solutions of iron-deficient plants resulted in a dramatic inhibition of Fe(III)HEDTA and Cu(II)Citrate reductase activity. The reductase activity in iron-deficient cucumber roots, measured with HCF(III), was inhibited to a lower extent after cupric ions treatment. On the other hand, the cupric-chelate Cu(II)HEDTA, applied at the same concentrations in solutions with (–Fe) plants, maintained the high stimulation of plasma membrane ferric-chelate and cupric-chelate reductase activity and produced additional acceleration of HCF(III) reduction by cucumber roots. The treatment with Cu(II)HEDTA improved the growth and root PMRA as well as other iron-deficiency stress responses of cucumber plants.
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KEY WORDS

cucumber (*Cucumis sativus* L.),
iron deficiency
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hexacyanoferrate(III) reduction

Copper and iron as important micronutrients with similar redox-properties need strict control in their mobilisation, uptake and translocation. The regulation of Fe and Cu homeostasis in plant cells under non-optimal growth conditions is extremely important for both plant productivity and human nutrition. Iron deficiency chlorosis as a limiting factor for plant growth and yield reduction is spread in different crops, mainly in alkaline carbonate soils, due to the insolubility of iron oxides and hydroxides (Schmidt 1999). Additionally, Fe-insufficiency stress can be combined with increased level of copper moving in plant tissues by fungicide spraying against diseases (Babalakova et al. 2003). In conditions of iron deficiency dicotyledonous plants such as cucumber develop various adaptive morphological and biochemical mechanisms to improve iron acquisition in soil solutions (Fe-deficiency stress responses; Raboti et al. 1995; Espen et al. 2000). The main adaptive process includes a strong increase in plasma membrane (PM) ferric-chelate reductase activity by roots accompanied with enhanced proton release needed for the reduction of Fe(III) to more soluble Fe(II) in the apoplast. Acidification of the rhizosphere is fulfilled by activation of PM proton pump and the biosynthesis of specific ferrous transporters at PM is accelerated (Robinson et al.1999; Schmidt 1999; Curie and Briat 2003). The PM-associated ferric-chelate reductase is the most studied redox enzyme; it is an integral membrane protein belonging to a family of flavoproteins that transfer electrons from cytosolic NADH to extracellular electron acceptors via FAD and heme groups (Robinson et al. 1999; Curie and Briat 2003). Besides a high induction of ferric-chelate reductase activity (FeChRA) in roots of iron-deficient plants, the reduction capacity for cupric compounds as well as hexacyanoferrate(III) reductase activity [HCF(III) RA] was also stimulated by iron deficiency, however, their relation to the uptake of copper and iron is not clear (Welch et al.1993; Babalakova and Schmidt 1996; Holden et al.1996; Weger 1999). The enhanced activity of the root PM redox system might contribute to cation uptake alteration under conditions of iron deficiency. Thus comparison of ferric- and cupric-chelate reduction by intact cucumber plants deserved attention. It was established that iron deprivation brought to increased content of copper in roots (Herbik et al.1996), but was also shown that ionic copper produced an inhibition of both induction and function of FeChRA in roots of iron-deficient plants (Alcantara et al.1994; Romera et al.1997; Schmidt et al.1997; Cohen et al.1998). However, it is not clear whether or not cupric chelate affects the regulatory control of the response to Fe-deficiency. In our earlier study we have established that short-term application of chelated copper, Cu(II)HEDTA, sustained the high activity of ferric-chelate reductase in roots of iron-deficient cucumber plants,

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kept the proton release and additionally stimulated cupric-chelate and HCF(III) RA. These effects were opposite to the strong inhibitory action of free copper ions, applied at the same concentrations in solutions of iron-deficient plants (Babalakova et al. 2005). The aim of the present study was (i) to investigate the influence of longer-term application (11-12 days) of Cu(II)HEDTA and free Cu-ions in the nutrient solution of control (+Fe) and Fe-deficient (–Fe) cucumber plants, (ii) to follow the growth and reactions of plasma membrane ferric-and cupric-chelate RA in roots of intact cucumber plants, and (iii) to study the activity of standard PM redox system (with HCF(III) as electron acceptor) in control and iron-deficient plants.

Materials and Methods

Plant material

Seeds of cucumber (*Cucumis sativus* L. cv. Gergana) were germinated in Petri dishes on filter paper moistened with 0.1 mM $CaCl_2$ in the dark at 28°C for 3 days. After 3 days the seedlings were transferred to grow in Hoagland-Arnon I nutrient solution (pH 6.0) in plastic pots in an environmental chamber. The nutrient solution was changed every second day and supplemented with 20 μM Fe(III)HEDTA [Fe(III) complex of N-(2-hydroxyethyl) ethylenediamine triacetic acid] and 0.2 μM of $CuSO_4$ for control (+Fe) plants (Babalakova et al. 2004). After receiving one-tenth concentration of the nutrient solution, Fe-deficient cucumber plants were grown in such solutions without Fe. Cucumber plants were harvested 12-13 days after the start of treatments, and morphological parameters – root, stem and leaves fresh weight (FW) – were measured and the reductase activities (RA) of roots of intact plants were determined.

Long-term treatment of control and Fe-deficient plants with ionic and chelated copper

Four-day-old seedlings were treated with different concentrations of $CuSO_4$ (0.2, 2, 10 and 20 μM) or Cu(II)HEDTA (0.2, 2, 20 and 100 μM; prepared as a stock solution, pH 6.0, at ratio of Cu to HEDTA of 1:1.25 as Tris-KOH complex). Treatment solutions were changed on every other day for twelve days. Fe(III)HEDTA used for (+Fe) plants was prepared as a stock solution, at ratio of $FeCl_3$ to HEDTA of 1:1.25 as Tris-KOH complex, pH 5.5. The chemical forms of applied copper, used to compare their effects in control (+Fe) and Fe-deficient (–Fe) plants, have different electrical charges. Copper in copper(II) sulfate forms a cupri-hexahydrate cation, $[Cu(II)(OH_2)_6]^{2+}$, in aqueous solution and keeps the ionic properties of copper. When HEDTA is added to aqueous solution of copper(II) sulfate, HEDTA forms a strong chelate with Cu(II), hydroxyethyl ethylenediamine-triacetato cuprate $[Cu(II)HEDTA]^{2-}$, with anionic character (Coombes et al. 1978).

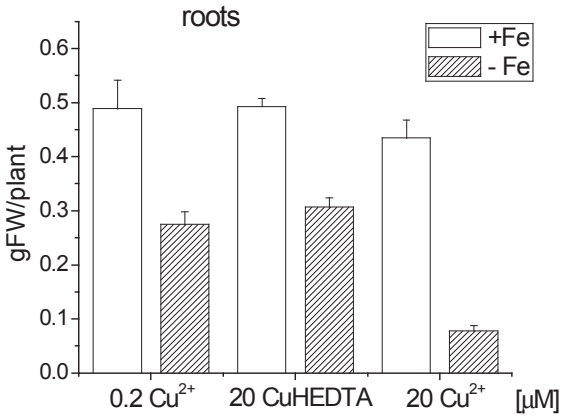

Figure 1. Influence of treatment with ionic copper (0.2 μM and 20 μM Cu^{2+}) or chelated copper (20 μM CuHEDTA) on the root fresh weight of control (+Fe) and Fe-deficient (–Fe) cucumber plants.

Measurement of ferric- and cupric-chelate reductase activity by intact roots

Fe(III)HEDTA and Cu(II)Citrate (as a more natural substrate) were used as electron acceptors. Cupric citrate was prepared at ratio of $CuCl_2$ to Citrate 1:3 as Tris-KOH complex, pH 6.5. The incubation medium for reductase activity measurements contained 0.1 mM $CaCl_2$, 0.15 mM Fe(III) or Cu(II) complex and 0.3 mM BPDS or BCDS in a final volume of 10 or 15 ml in dark vessels at pH 5.5 for Fe(III)ChRA and pH 6.5 for Cu(II)ChRA as described previously (Babalakova and Schmidt 1996; Babalakova and Traykova 2001). The reductase activity of intact roots was expressed in μmol $Fe(II)\cdot g^{-1}$ root $FW\cdot h^{-1}$ or $Cu(I)\cdot g^{-1}$ root $FW\cdot h^{-1}$. HCF(III) RA was performed according to Schmidt (1994).

Statistical analysis

The experiments were repeated at least 3 times with 6 to 8 intact plants in each variant. The data presented are the average of 18 to 24 samples and the values in the figures and tables represent the standard errors of the mean values. Differences between variants were compared by Student's *t*-test at 5% level of significance.

Results

Two-weeks-old control cucumber plants (+Fe; 0.2 μM Cu^{2+}) had expanded green cotyledons and first and second truly green leaves. Cucumber plants grown 11-12 days without Fe in the nutrient solution developed leaf chlorosis. Reduction of the root growth with morphological changes, characteristic of iron deficiency, was also observed. Depending on the chemical form of used copper, the decrease of cucumber root fresh weight (RFW) under conditions of iron starvation was dif-

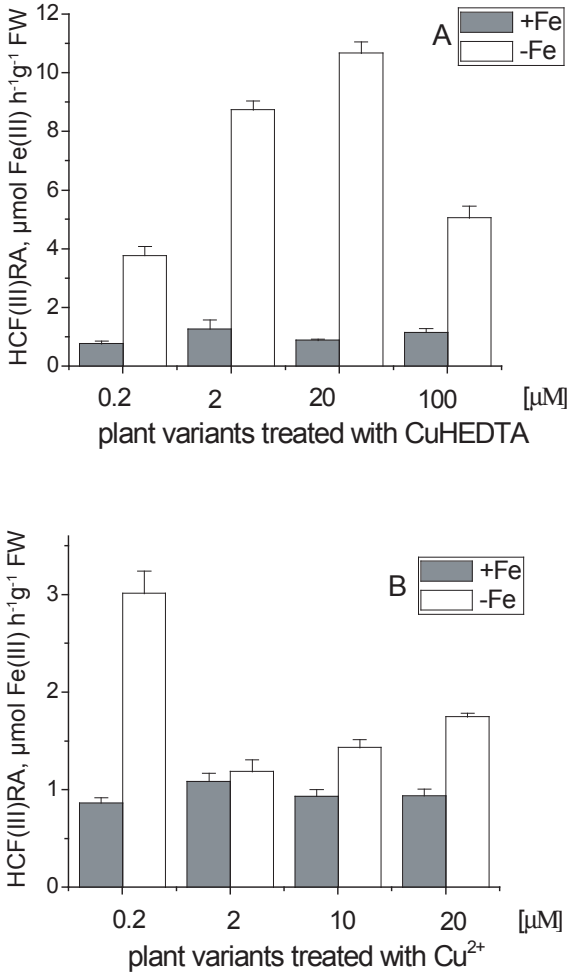

Figure 2. Changes in hexacyanoferrate(III) reductase activity [HCF(III)RA] at root plasma membrane of cucumber plants grown in conditions of normal iron nutrition (+Fe) or under iron deficiency (–Fe), and treated with various concentrations of cupric-chelate (CuHEDTA; A) or free cupric ions (Cu^{2+}; B).

ferent (Fig. 1). Fe-deficiency in solutions containing control level of copper (0.2 µM) provoked about 45-50% reduction of RFW of (–Fe) plants as compared to (+Fe) plants. The application, however, of 20 µM free copper ions for 12 days resulted in dramatic inhibition of the growth and mass production of Fe-deficient cucumber plants. RFW was reduced by about 85% and plants remained small and undeveloped. The same concentration of Cu(II)HEDTA (20µM) markedly improved the growth of entire cucumber plants under conditions of Fe-shortage and remedied RFW of (–Fe) plants about 3.5 to 4 fold during the experimental period (Fig. 1). The continuous treatment with cupric chelate was followed with decrease of leaf chlorosis (data not shown). Micromolar concentrations of copper used (ionic or chelated) did not or only slightly influenced the growth of control (+Fe) plants. We have chosen the concentration of 20 µM cupric chelate to show marked growth improvement of Fe-deficient cucumber plants, as compared to the high inhibitory growth at the same concentration of ionic copper (Fig. 1). On the other hand, (–Fe)-plants treated with 2 µM Cu^{2+} developed very strong chlorosis but growth of leaves was not inhibited, while the growth of plants treated with 20 µM Cu^{2+} were strongly inhibited.

Under conditions of iron deficiency, considerable enhancement of ferric- and cupric-chelate RA was found in cucumber roots (Table 1; about 7- fold increase in (–Fe)-plants). Cupric-chelate reductase activity was measured with Cu(II)Citrate; Cu(II)Citrate is a more natural substrate but chemically it is a weak chelate and more stable at alkaline pH (6.5 was used by us). Added Tris stabilized the complex. However, ferric-chelate reductase activity was measured with Fe(III)HEDTA, a stable chelate at the pH optimum of 5.5. Both reductase activities of intact cucumber roots showed similar increase or decrease depending on the chemical forms of applied copper (Table 1). It was supposed that reduction of ferric- and cupric-chelates by dicotyledonous plants could be performed by the very same membrane reductase (Welch et al.1993). A high inhibition of Cu(II)Citrate RA in (–Fe) plant roots, with respect to their (–Fe) control, was registered after continuous treatment with ionic copper (2 µM and 20 uM). This activity became smaller than the control activity in (+Fe) plants (about 20% and 50% inhibition; Table 1). The Fe(III)HEDTA reductase activity was inhibited to even higher extent than CuChRA by free copper ions (Table 1). Increasing the external copper concentration to 20µM resulted in further decrease of the reduction rate of both reductases. Application of the same concentrations of cupric chelates in solutions of iron-deficient plants, however, brought to considerable stimulation of reductase activity with both electron acceptors. Cu(II)HEDTA applied in nutrient solutions of (–Fe) plants at concentration of 20 µM provoked about 2.2 - 2.4 fold additional activation of cupric- and ferric-chelate RA. The reductase activities in variants of (+Fe) roots were slightly changed after continuous treatment with copper ions or chelate (Table 1).

Standard (or constitutive) redox system activity at PM of cucumber roots (measured usually with HCF(III) as an electron acceptor) reacted to Fe-starvation by 3-fold increase of reduction rate in comparison to control (+Fe) activity (Fig. 2A and 2B). Continuous supply with Cu(II)HEDTA markedly accelerated HCF(III) RA in Fe-deficient cucumber roots, proportionally to the increasing concentrations of cupric chelate from 0.2 to 20 µM (Fig. 2A). Treatment with higher concentrations of Cu(II)HEDTA (100µM) caused less increase in the activity but kept it higher with respect to that of the (–Fe) control plants. The application of free cupric ions (2, 10 or 20 µM) in nutrient solutions of iron-deficient cucumber plants resulted in about 50% inhibition of HCF(III)

Table 1. Alteration of cupric(A)- and ferric(B)-chelate reductase activity in roots of Fe-sufficient (+Fe) and Fe-deficient (–Fe) cucumber plants after continuous application of ionic (Cu^{2+}) or chelated (CuCh) copper in the nutrient solution.

VARIANTS (A)	Cu(II)Citrate RA [µmol Cu(I) . g^{-1} FW . h^{-1}]		% -Fe/+Fe
	+Fe	- Fe	
0.2 µM Cu^{2+}	0.158 ± 0.020	1.208 ± 0.093	765
0.2 µM CuCh	0.165 ± 0.019	1.464 ± 0.101	887
2 µM Cu^{2+}	0.174 ± 0.016	0.138 ± 0.006	79
2 µM CuCh	0.181 ± 0.019	1.693 ± 0.071	935
20 µM Cu^{2+}	0.188 ± 0.020	0.102 ± 0.011	54
20 µM CuCh	0.130 ± 0.015	2.204 ± 0.138	1695

VARIANTS (B)	Fe(III)HEDTA RA [µmol Fe(II). g^{-1} FW . h^{-1}]		% -Fe/+Fe
	+Fe	- Fe	
0.2 µM Cu^{2+}	0.271 ± 0.052	1.982 ± 0.152	731
0.2 µM CuCh	0.275 ± 0.051	2.498 ± 0.125	908
2 µM Cu^{2+}	0.276 ± 0.050	0.153 ± 0.009	55
2 µM CuCh	0.221 ± 0.023	2.786 ± 0.142	1261
20 µM Cu^{2+}	0.285 ± 0.030	0.045 ± 0.001	16
20 µM CuCh	0.175 ± 0.015	3.093 ± 0.165	1767

RA with respect to (–Fe) plants, but kept it at higher values than the activity was in (+Fe) plants (Fig. 2B).

Discussion

Despite intensive research, the role of chelation in the mechanisms of metal uptake, translocation and metabolism in plants is yet not properly understood. The reactivity of copper ions to form stable complexes and to participate in redox reactions at the plasma membrane put forward the conception that copper can displace iron from Fe(III) complexes in nutrient solutions with iron supply (Guinn and Joham 1963; Taylor and Foy 1985). Data describing what might be the plant reactions towards application of cupric-chelates in the absence of iron are not available. Our results showing the improved growth of (–Fe) cucumber plants (Fig. 1) and the ameliorating effect towards leaf chlorosis (data not shown) after the treatment with cupric chelate do not support the suggestion that the primary toxic effect of Cu(II)EDTA could be the induction of iron deficiency in plant leaves (Taylor and Foy 1985). These authors used, however, very high concentrations of cupric chelate. Also, the increase of HCF(III) RA in roots of (–Fe) plants as well as the considerable stimulation of ferric- and cupric- reductase activity (Table 1 and Fig. 2) correlates with remedying growth after application of cupric chelates. These results are in good agreement with the conception that acceleration of PM redox activity can be directly implicated in the regulation of plant growth (Doering et al. 1998).

Some controversial data exist about the extent of uptake of ionic or chelated elements. It has previously been shown that accumulation of copper might be markedly affected by the chemical form of the applied copper, depending on the charge of Cu-complexes. The comparison of the uptake patterns of a positive copper complex with that of an anionic Cu-complex demonstrated that Cu(II)EDTA was accumulated poorly (Coombes et al. 1977, 1978). Recently, the results of Schmidt et al. (1997) have underlined that both ionic Cu and Cu(II)EDTA can be readily transported through the plasmalemma of root cells. The uptake of intact chelate molecules has already been reported for Cu and Pb (Bell et al. 1991; Vassil et al. 1998).

It was supposed for dicotyledonous plants that reduction of ferric- and cupric-chelates could be performed by the very same membrane reductase (Welch et al. 1993). Later investigations were in accordance with the presence of various redox proteins at the plasma membrane of different organisms that can act as cupric- and ferric-chelate reductases (Babalakova and Schmidt 1996; Holden et al. 1996; Weger 1999). The pH optimum was also different for the two reductases. Other results suggested that the ferric-chelate reductase activity, measured at pH 5.5, in Fe-deficient plants (the "turbo" reductase) might be different from the constitutive redox activity measured with HCF(III) as electron acceptor (Babalakova and Schmidt 1996; Holden et al. 1996; Susin et al. 1996). Despite using different substrates and treatments in our experiments, Fe deficiency caused similar changes in the cupric- and ferric reductase activities and demonstrated the presence of redox proteins with similar properties at PM. High activation of cupric reductase in the roots of Fe-deficient plants might be connected to the same extent with increased copper uptake under iron starvation (Herbic et al. 1996). The direct connection between enhanced cupric-chelate reduction and increased copper content in plant roots, however, is not clear. The induction of both FeChRA and HCF(III) RA under iron starvation in different plants varied to different extent because of enzyme heterogeneity (Schmidt 1994; Lynnes et al. 1998).

The considerable inhibitory effect of ionic copper on the plant root reducing capacity after creation of Fe deficiency confirmed previously obtained results (Alcantara et al.1994; Romera et al. 1997; Schmidt et al. 1997). The alteration of RA in Fe-deficient plants was related to pH changes of the nutrient solutions during iron starvation and copper treatment. Application of ionic copper started to inhibit release of protons by roots of Fe-deficient plants from the first day of solution change (Babalakova et al. 2005) and this inhibition correlated with the high inhibition of FeChRA by ionic copper. At the same time chelated copper application stimulated the H^+ extrusion by the roots of Fe-deficient cucumber plants. Enhanced acidification of the medium during iron starvation is important for the induction of and sustaining the high level of FeChRA in many plants, because the enzyme is pH sensitive (Wei et al. 1997; Schmidt 1999). It has recently

been proved that high apoplastic pH depressed FeChRA and restricted the uptake of Fe(II) into the cytosol (Kosegarten et al. 2004). Thus, one of the possible explanations for the high inhibition of ferric-reduction by free copper ions in roots under Fe-deficiency is the inhibition of proton release. Our results showed a correlation between proton release and stimulation of FeChRA under conditions of iron starvation. It was shown in some publications that the rate of cupric reduction was a function of the free Cu^{2+} as an actual substrate for cupric reductase activity (Holden et al. 1996; Weger 1999). Our results demonstrated that cupric-chelate reductase at the plasma membrane of Fe-deficient cucumber roots expressed a higher activity in the presence of chelated copper. Another possible explanation for the inhibitory effect of ionic copper towards FeChRA in iron-deficient roots is based on the assumption that Cu^{2+} might act as a powerful scavenger of the superoxide radical, which was shown to facilitate Fe-chelate reduction at the plasma membrane (Cakmak et al. 1987; Macri et al. 1992). This suggestion is supported by the experiments with in vitro application of copper ions that produced the inhibition of Fe(III)EDTA RA in (–Fe) plants already within the first minutes (Schmidt et al. 1997). Another effect of ionic copper, inhibiting to a higher extent the proton release in (–Fe) cucumber plants, might be the reduced activity of the plasmalemma proton pump (Babalakova and Hager 1994). Ionic copper could also affect Fe nutrition by inhibition of some components or a subunit of the trans-plasma membrane electron transport chain. In the presence of chelating agents, copper ions form chelates that can be taken up by plant roots (Schmidt et al. 1997). These authors showed that at equimolar Fe and Cu levels, *i.e.* in the presence of Cu chelates, induction of the iron-stress response was not inhibited, thus supporting our results for stimulating effects of cupric chelates on the FeChRA in Fe-deficient cucumber plants.

The exact site of copper action remains to be established. In spite of the intensive research, (i) the role of chelation itself, (ii) the role of different metal complexing agents with different charges in the mechanisms of metal uptake by plants, and (iii) the action of metal complexes in induction of RA at membrane level are still poorly understood. The high increase of standard redox system activity (measured as HCF(III) RA) in Fe-deficient cucumber roots upon application of cupric chelates demonstrated strong enhancement of trans-membrane electron transport that might be also connected with sustained activity of the proton pump. Interactions between the H^+-ATPase activity and the redox state of the cytoplasm have been suggested to play an important role in the regulation of electron transport by the standard redox system (Schmidt 1999). On the other hand, we supposed that treatment with copper chelate might induce the biosynthesis of phytocyanines (or cupredoxins), low-molecular electron–transporting proteins in the plasma membrane that contains copper (Nersissian et al. 2001). Cucumber plants are rich of stellacyanine and we hypothesised on a plausible role of cupredoxins in facilitating the transport of electrons to standard redox system in roots that could explain the high increase of reductase activity. What will be the exact explanation for the reaction of iron-deficient plants with increased reductase activity only under influence of chelated copper, needs further research. As the chelated copper loses its charge or ionic properties, the first place of action could be the plasma membrane of root cells. Further investigations are needed to resolve the problems of how ionic and chelated copper acts at membrane level in (+Fe) and (–Fe) cucumber plants as well as how Cu and Fe is metabolised and translocated to shoots.

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