Copper and photosystem II: A controversial relationship

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Abstract

Copper is an essential micronutrient for higher plants and algae and has a direct impact on photosynthesis. It is a constituent of the primary electron donor in photosystem I, the Cu-protein plastocyanin. Many authors have also described Cu as a constituent of photosystem II (PSII). However, high Cu concentrations inhibit the photosynthetic electron transport, especially in PSII. In addition, both Cu deficiency and Cu toxicity interfere with pigment and lipid biosynthesis and, consequently, with chloroplast ultrastructure thus negatively influencing the photosynthetic efficiency. In this review, the different functions proposed for the metal in PSII are reviewed. With reference to the effect of toxic Cu on PSII, the polemic results concerning its mechanism of action and Cu-binding sites are discussed. Other effects of Cu toxicity and Cu deprivation on the thylakoid membrane are also briefly described.

Key words - Copper deficiency, copper toxicity, photosystem II.

Abbreviations - BBY, PSII preparations isolated as described by Berthold et al. (1981); BBY_{s+} and BBY_{s-}, starch-containing and starch-tree BBY isolated according to Arellano et al. (1994); CP26, minor antenna complex of PSII; DCBQ, 2,6-dichloro-benzoquinone; DPC, diphenylcarbazide; EPR, electron paramagnetic resonance; FeCN, ferricyanide; F_v, variable fluorescence; LHCII, major light-harvesting complex of PSII; (Mn_x), manganese-containing water oxidase; OEC, oxygen-evolving complex; P680, reaction center chlorophyll(s) of PSII; Phe, pheohytin; PS, photosystem; Q_A, Q_B, primary and secondary quinone acceptors of PSII; SiMo, silicomolybdate; S-states, redox states of the oxygen-evolving complex; Tyr_z, redox active tyrosine 161 of the Dl protein.

Introduction

Many stress factors of higher plants, such as excess light, drought, salt, heat, cold, pathogens, or heavy metals, produce primary deleterious effects on photosynthesis. Photosystem II (PS II), mainly located in the appressed regions of the thylakoid membranes, the grana stacks, is the most sensitive target site for the metabolic disturbances induced under conditions of stress. The structural heterogeneity and peculiar metabolism associated with PSII (for review see Andersson and Styring 1991) facilitate a relatively fast adjustment to changing environmental conditions, as well as rapid repair of the damaged structures. The mechanisms behind the stress-induced damage are currently an area of intense research and parallel the increasing knowledge of the system.

Certain metals, such as Cu, are constituents of a great number of proteins and enzymes and are consequently essential for maintaining optimum plant metabolism (for review see Sandmann and Böger 1983). Copper deficiency has a direct impact on energy metabolism because it affects the synthesis of the Cu-containing electron carriers plastocyanin and cytochrome oxidase, whose depletion results in decreased photosynthesis and respiration (Walker and Webb 1981, Baron and Sandmann 1988a,b).
The sensitivity of the photosynthetic apparatus to excess Cu was first demonstrated by Macdowall (1949) and the inhibitory effect of Cu on both photosystems has since been confirmed in a number of publications (for review see Droppa and Horvath 1990). This effect of Cu is of interest as this element has become a widespread pollutant due to its use as algicide and fungicide in agriculture.

Despite their consequence for plant productivity and the environment, the physiological function of some metals in PSII and their mechanisms of action at toxic concentrations are still not fully understood. One exception could be Mn, which plays a key role in the water splitting system. Regarding Cu, both its mechanism of action and involvement in the function of PSII remain controversial.

In order to better understand the process by which Cu depletion and toxicity affect PSII function, we summarize and discuss the various roles attributed to Cu in PSII, as well as the proposed sites of Cu inhibition. We also briefly describe the additional effects on PSII of an anomalous mineral nutrition.

Presence of Cu in PSII preparations

The different procedures for obtaining oxygen-evolving PSII preparations are based mainly on detergent solubilization of the thylakoid membrane. Anderson et al. (1964) were the first to find Cu in the PSII-enriched fraction obtained from digitonin-fractionated spinach chloroplasts. When the method described by Berthold et al. (1981) became a standard procedure for PSII isolation, Cu was detected in BBY preparations isolated from different plants. Table 1 shows the high variability of the Cu content, reported in this type of preparation. Cu values of 4.2 atoms per 300 Chl molecules in wheat (Sibbald and Green 1987), 3.3 in pea (Baron et al. 1992) and 2.2 in *Vicia faba* (Gol´dfel´d and Khalilov 1979) were obtained. Surprisingly, Droppa and Horvath (1990) reported amounts of 5.7, 2.2, 2.5, 1.8 and 1.2 atoms Cu per 300 Chl molecules in spinach. Arvidsson et al. (1993) isolated BBY from spinach with a Cu content that ranged between 0.1 and 1.0 atoms per 300 Chl

molecules. Some of the authors attributed these substantial variations to loss of Cu during the preparative procedures. However, Baron et al. (1993) proposed that certain steps in the isolation of PSII-enriched particles (BBY) influenced the Cu content of the final preparation. Centrifugation at 10000 g, used to remove starch after Triton-treatment of the thylakoid membranes, yielded starch-free BBY (BBY_{S-}) preparations of low Cu content. In contrast, starch-containing BBY (BBY_{S+}) had about 2 atoms Cu per 300 Chl, in both spinach and pea (Arellano et al. 1992, 1993). It was demonstrated that histones, nucleic acids and some metal ions appeared in the starch pellet after centrifugation. The authors proposed that Cu is associated with this nuclear contamination (Arellano et al. 1994) and could be easily removed from the PSII complex by eliminating starch, with no negative effects on the oxygen-evolving activity. Moreover, they recommend use of a preparation similar to their BBY_{S-} to obtain reliable results of the interaction of metals with PSII.

Role of Cu in the regulation of PSII electron transport

The detection of Cu in PSII preparations first suggested that the metal was either a PSII constituent or in some way involved in PSII-linked electron transport. The search for a putative Cu-binding protein in this photosystem suggested to some authors that Cu could be associated to the antenna complexes (see Tab. 1). Sibbald and Green (1987) reported that about 75 of the Cu in PSII preparations from barley and spinach was bound to the major antenna complex of PSII (LHCII). Later, Droppa and Horvath (1990) speculated that Cu might be involved in protecting the major antenna from damage by the deleterious radicals formed during chloroplast function. Both authors had earlier detected a reduction

of a 29 kDa polypeptide in severely Cu-deficient chloroplasts, suggesting that this was a minor antenna complex of PSII. Arvidsson et al. (1993) claimed that Cu was a constituent of the minor antenna complex CP26, and proposed that this could be the missing oxidase of the xanthophyll cycle. Early experiments with Cu-chelators had suggested a functional role for Cu in PSII-mediated electron transport (see references in Droppa and Horvath, 1990). However, the uncertainty regarding the action sites of these chelators made it difficult to assess a specific role for the metal.

The involvement of Cu in the water-splitting system was first assumed because its presence in Tris-washed potato tuber chloroplasts resulted in the recovery of oxygen evolving activity (Ramaswamy and Madhusudanen 1978). Holdsworth and Arshad (1977) isolated a Mn-Cu-protein complex from the diatom, *Phaeodactylum* tricornutum (see Cu content in Tab. 1), presumptively involved in water oxidation. Ono et al. (1984) found that Cu could be washed out of PSII particles, and that changes in the oxygen-evolving ability were correlated with variations in the Cu content of the preparation.

The study of Cu deficiency in higher plants and algae has been a successful experimental approach in the analysis of the functional involvement of Cu in PSII-mediated electron transport. Inhibition of the electron transfer in PSII has been determinated in Cu-deficient plants of *Zea mays* (Barr and Crane 1974), *Pinus radiata* (López Gorgé et al. 1985, Lastra et al. 1987), *Pisum sativum* (Baron and Sandmann 1988a, Barón et al. 1990, 1992), *Spinacia oleracea* (Droppa et al. 1987), *Beta vulgaris* (Horvath et al. 1983) and in the green alga *Dunaliella*, grown under conditions of Cu-deficiency (Sandmann 1985). The latter author attributed this effect to the partial damage of the photosynthetic apparatus, by low

quantities of the different electron carriers and not to a specific inhibition of PSII induced by Cu-deficiency. Droppa et al. (1987) and Baron et al. (1992) suggested that Cu-deficiency influenced electron transport in PSII through changes in its lipid microenvironment. They found a tendency in Cu-deprived plants towards an increase in saturated fatty acids of the thylakoid lipids, mainly in phospholipids, which play a regulatory role in PSII function. According to other authors, a reduction in levels of the photosynthetic pigments, chlorophyll and carotenoids, as well as in those of the lipids, could also explain the decreased PSII electron flow caused by Cu deprivation. Baron et al. (1992) demonstrated a decrease in carotenoids, and a variation in the xanthophyll distribution in pea LHCII from Cu-deficient plants. Baszynski et al. (1978), Horvath et al. (1983) and Henriques (1989) proposed that Cu deficiency reduces the amount of photosynthetic pigments interfering in the early steps of terpenoid biosynthesis.

Experiments carried out with spinach and sugar beet grown under severe Cu-deficiency were not able to support the assumption that Cu is involved in water oxidation. Horvath et al. (1983) found that the inhibited PSII electron transport in such plants could not be re-stored by the addition of artificial electron donors such as diphenylcarbazide (DPC) and NH_2OH, both of which donate electrons after the water oxidation system (see Fig. 1). By measurements of fluorescence induction and analysis of thermoluminescence curves in Cu-deficient chloroplasts, they proposed that Cu-deprivation affected the PSII acceptor side, similar to other DCMU-like inhibitors. These authors suggest that the Cu-induced changes in the lipid composition of PSII disturb the microenvironment of the Q_B site.

Tab 1. Cu content (Cu atoms per 300 Chl molecules) in PSII preparations isolated from different species and percentage of PSII Cu bound to antenna complexes. -, Not determined.

	PSII	*LHCII*	*CP26*
Phaedactylum tricornutum	1.3	-	-
Vicia faba	2.2	-	-
Triticum aestivum	4.2	75	-
Pisum sativum	3.3	50	-
	2.0 (BBY_{s+})	-	-
	0.2 (BBY_{s-})	-	-
Spinacia oleracea	5.7	-	-
	2.2	-	-
	1.2	75	-
	2.5	75	-
	1.9	78	-
	0.1-1.0	-	100
	2.3 (BBY_{s+})	-	-
	0.2 (BBY_{s-})	-	-

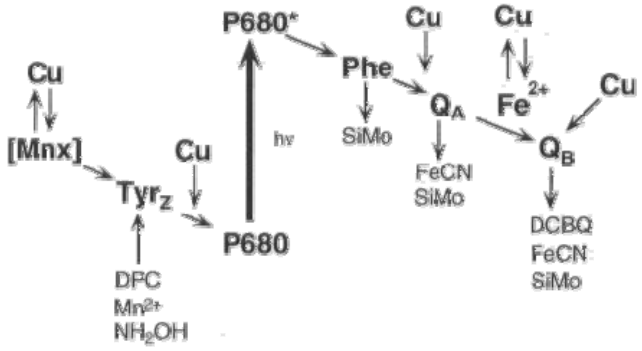

Fig. 1. Cu-inhibitory sites and action sites of different electron donors and acceptors in PSII-mediated electron transport.

Results are also contradictory regarding ultrastructural changes of the chloroplasts induced by Cu-deficiency. Vesk et al. (1966) and Droppa and Horvath (1990) found no change in the grana region, where most of PSII is located, whereas Henriques (1989) and Casimiro et al.(1990) have described a disorganization of the grana in Cu-deprived plants. The preparation of PSII-enriched BBY_{s-} particles, without nuclear contaminants, devoid of Cu and with high oxygen-evolving activity (Baron et al. 1993, Arellano et al. 1994), challenged the idea that Cu is a constituent of PSII. These authors demonstrated that Cu in PSII preparations is associated with nuclear contamination, and attributed the earlier reported presence of this metal and the ample variations in its content to an insufficient or unequal removal of this contaminant. Moreover, they explained the detection of Cu in the PSII antenna complexes LHCII and CP26 by the presence of unspecific metal-binding sites, where contaminant Cu could bind during the isolation process.

In summary, the most plausible conclusion in relation to the presence of Cu in PSII preparations and the effect of Cu-deficiency in PSII, is that Cu is not a constituent of PSII but may influence the PSII electron transport by controlling its lipid and pigment composition.

Inhibition by Cu of the PSII electron transport

Early studies have suggested that high Cu concentrations affect photosynthetic light reactions (Macdowall 1949), and that light is required for the expression of the toxic effect of Cu (Steemann-Nielsen et al. 1969, Cedeño-Maldonado and Swader 1972). The inhibition of PSI electron transport has been reported in algae and plants by various authors (Shioi et

al. 1978, Baszynski et al. 1988) and attributed to interactions in its reducing side, probably with ferredoxin. The cytochrome b_6/f complex has also been suggested as a Cu-inhibitory site (Singh and Singh 1987).

However, the interaction mechanism of Cu with PSII and the precise location of the binding site are still controversial (Fig. 1). The uncertainty concerning the action sites of some of the electron donors and acceptors used to test the Cu-inhibitory patterns, does not make the task any easier. In addition, some of the reagents and buffers used in such assays may interact chemically with Cu, thus producing unreliable results (Renganathan and Bose 1989).

Most authors locate the target of the Cu-inhibition of PSII to its oxidizing side. Samson et al. (1988) showed a fluorescence quenching effect when the copper concentration was increased in *Dunaliella tertiolecta* cultures. NH_2OH was found to be ineffective in recovering the variable fluorescence (F_v) and the rate of Q_A (primary quinone acceptor of PSII) photoreduction was not inacti-vated by Cu. Since NH_2OH inhibits the oxygen evolving complex (OEC) and acts as an electron donor, they sug-gested that Cu impaired the PSII photochemistry by an interaction at or beyond the PSII primary electron carrier donor, Tyr_z (redox active Tyr of the D1 protein, Fig. 1).

Shioi et al. (1978) indicated that Cu inactivates the PSII donor side after the DPC electron donating site, since this electron donor could not reverse the PSII inhibition by Cu in spinach and *Ankistrodesmus falcatus* chloroplasts. Vierke and Struckmeier (1977) pointed out that Cu could inhibit a component very close to the OEC through its binding to a residue of a membrane protein. Haberman (1969), Cedeño-Maldonado and Swader (1972),

and Samuelson and Öquist (1980) also stated that the oxidizing side of PSII was the most sensitive site for Cu-inhibition.

On the contrary, other studies conclude that Cu influences the PSII electron transport on the acceptor side. Yruela et al. (1991, 1993) proposed a target at the Pheo-Q_A-FC^{2+} domain. They found that the oxygen evolution by PSII membranes was inhibited by Cu when 2,6-dichloro-benzoquinone (DCBQ) or ferricyanide ($K_3Fe(CN)_6$), but not silicomolybdate (SiMo), were used as electron acceptors. A model was proposed according to which ferricyanide takes electrons from somewhere between the Q_B and Phe (pheophytin) sites, whereas DCBQ does so from the Q_B site, and SiMo from the reduced Phe.

Since the maximum yield of NH_20H fluorescence induction of Tris-inactivated thylakoids was noticeably reduced by Cu, Mohanty *et al*. (1989) suggested that Cu acts on the reaction center or on components beyond it. Furthermore, they found that Cu addition also diminished the Q_B component of delayed luminescence and the B thermoluminescence band which arises from the S_2Q_B charge recombination. Therefore, they proposed that Cu binds to the reaction center, thus inducing a structural alteration of the Q_B binding protein and loss of the Q_B function. They also considered the possibility that Cu may interact with the non-heme iron located in the vicinity of the Q_A and Q_B acceptors. Earlier results of Singh and Singh (1987) had already shown the restoration of Cu-induced inhibition of PSII activity after addition of Fe.

The PSII reaction center has also been considered as the Cu-inhibitory binding site. Hsu and Lee (1988) proposed that Cu created a lesion close to the reaction center, which

increases the probability of dissipation of the in-coming excitation energy. In their experiments, the initial fluorescence yield of thylakoids was hardly affected, whereas that of the F_v was lowered without significant kinetic change. The basic inhibition pattern was not altered by the addition of DCMU or by the abolition of the oxygen evolution capacity using Tris-treatment and NH_2OH as electron donor.

Renganathan and Bose (1989) found that Cu inhibited electron transport in $H_2O \rightarrow$ SiMo (+DCMU) photoreaction, as well as F_v in isolated reaction centers. The Hill reactions Mn $\rightarrow$ dichloroindophenol and Mn $\rightarrow$ SiMo (+DCMU) were inhibited in a similar manner in heat-treated thylakoids with an inactivated OEC. These au-thors found a residual F», which was not suppressed at those Cu concentrations that completely inhibited oxygen evolution. Such results were interpreted as an inhibition by Cu of the primary photochemistry of only a fraction of the PSII centers (PSIIα), while the others would be Cu insensitive.

Recent studies have reconciled the donor side and reaction center theories of the Cu target. They have attempted to locate the site of Cu inhibition in PSII by laser flash-induced absorption spectroscopy (Schroder et al.1995), in addition to the most commonly used polarographic and fluorescence methods (J. B. Arellano, J. J. Lázaro, J. Lopez Gorge and M. Baron, unpublished results). They showed that Cu inhibited the DCMU-in-sensitive $H_2O \rightarrow$ SiMo activity of PSII particles and decreased F_v of Tris-washed preparations with Mn as electron donor. They located the Cu target on the donor side, close to the reaction center. Like Renganathan and Bose (1989) the authors found a Cu-insensitive component of F_v, suggesting that Cu inhibits only a fraction of the PSII centers

which contribute to F_v but do not participate in Q_B reduction. Schroder et al. (1995) measured laser flash-induced absorption changes at 830 and 436 nm and observed that the kinetic of $P680^+$ reduction in isolated PSII particles, reaction centers and Tris-washed PSII particles, was markedly slower in the presence of Cu. A detailed analysis of the different components of these kinetics showed that Cu specifically inhibited the electron donation from Tyr_z to $P680^+$, either by a modification of this amino acid in the D1 protein and/or its microenvironment. The extent of $P680^+$ and Q_A formation under repetitive flashes measured at 325 nm showed unambigously that Cu does not affect the primary charge separation. However, they did not exclude Cu-induced secondary effects near the Q_B site, reflected in the variation of the affinity to herbicide binding after Cu-treatment (Renger et al, 1993).

J. B. Arellano, C. Jegerschöld, W. P. Schroder, M. Baron and S. Styring (unpublished results) confirmed by means of electron paramagnetic resonance (EPR) spectroscopy studies that Cu blocks the electron transfer $Tyr_z \longrightarrow P680^+$. In the presence of Cu, the multiline signal of the S_2 state of the OEC was abolished. In addition, the signal $II_{very\ fast}$ and signal II_{fast} both reflecting oxidised Tyr_z in intact BBY and Tris-washed BBY preparations, respectively, were inhibited and a new radical was oxidised by $P680^+$ In addition, it was shown that the socalled Q_A-Fe^{2+} EPR signal was not dramatically changed by Cu, indicating that the charge separation remained functional as previously suggested by Schroder et al. (1995).

Early studies have shown that PSII was more sensitive to toxic Cu concentrations at high pH (Steemann-Nielsen et al. 1969). Vierke and Struckmeier (1977) tested the pH

dependence of the Cu-inhibition luminescence curve in spinach chloroplasts, and found that a protonation equilibrium was involved in the Cu-binding to a dissociated residue of a membrane protein on the PSII donor side. The EPR spectra showed that the four ligands for Cu are three atoms of oxygen and one of nitrogen. Later experiments carried out by Yruela et al. (1992), demonstrated a competitive inhibition by Cu of proton-binding. More recently, they suggested an interaction of Cu with some amino acid residues (His or Tip) located on the pheophytin-Q_A domain, which can be protonated and deprotonated. This could disrupt the local conformation and thus inhibit photosynthetic electron transfer.
J. B. Arellano, J. J. Lázaro, J. Lopez Gorge and M. Baron (unpublished results) have also tested the influence of pH on the inhibition by Cu of the fluorescence induction kinetics of Tris-washed PSII particles with an inactivated OEC. They suggest that Cu and FP compete for the same ligand on the PSII donor side.

The reversibility and light dependence of Cu action on PSII have also been controversial. Steemann Nielsen et al. (1969), and Cedeño-Maldonado and Swader (1972) proposed that the toxic effect occurs only in light. Haber-man (1969) and Gupta (1986) showed a reversibility by addition of Mn, whereas Hsu and Lee (1988) found a restoration of activity after elimination of excess Cu. Renganathan and Bose (1989) associated this effect to a binding of Cu by the buffers and reagents used to test Cu toxicity, from which it is then prevented of reaching its target. Cedeño-Maldonado and Swader (1972) and Samuelson and Öquist (1980) proposed that the inhibition of the electron transport induced by Cu was irreversible. Experiments on the reversibility by EDTA of the Cu-induced inhibition, carried out by J. B. Arellano, J. J. Lázaro, J. López Gorgé and M. Barón

(unpublished re-sults), showed that Cu provokes irreversible damage which is exacerbated by light.

From the experiments reviewed it can be concluded that the main effect of excess Cu on PSII is an irreversible donor-side inhibition. This does not exclude Cu interfering with components on the acceptor side, near Q_B.

Other effects of Cu interference in PSII: Changes induced by Cu deficiency and Cu toxicity in chlo-roplast ultrastructure and in lipid and pigment composition

Uptake of Cu in vivo by higher plants and algae, as well as its deprivation in culture media, can affect some other plant processes. In chloroplasts, these anomalous situations can disturb the architecture of thylakoid membranes which, in turn, affect some light reaction processes, especially those associated with PSII. At this point, it is essential to know whether the alterations in chloroplast fine structure, as well as changes in pigment and lipid composition, are responsible for the short- and long-term effects induced by this metal. In a preceding section we considered whether or not Cu deprivation affects the structure of the grana. It is evident that excess Cu has a strong effect on chloroplast fine structure, resulting in a degradation of grana stacks and stroma lamellae, and an increase in the number and size of plastoglobuli and intra-thylakoidal inclusions (Baszynski et al. 1988). Sandmann and Böger (1980) correlated these disturbances with processes of enhanced lipid peroxidation. The release of free fatty acids from lipid degradation influences the reactions of electron transport (Schroder et al. 1992), mainly on the water oxidation side (Maksymiec et al. 1992). Maksymiec et al. (1992) showed, in Cu-treated

plants, an increase in the saturated fatty acids of the different lipid categories, as well as a lower ratio of monogalactosyldiacylglycerol to digalactosyldiacylglycerol, which indicates a decrease in grana stacking. Smith et al. (1985) suggested that Cu might interfere with the unsaturation and elongation processes of lipids, both in brown and red algae.

We have already stated that in Cu-deficient chloro-plasts the lipid composition was altered (Droppa et al. 1987); phospho- and sulfolipid contents were increased, while those of galactolipids were reduced. In addition, the fatty acid unsaturation increased in galactolipids and decreased in sulfo- and phospholipids. The fact that lipids are effective in regulating PSII electron transport has been demonstrated by other authors (e.g., Tremolieres and Gamier 1990).

The reduction of photosynthetic pigments induced by Cu excess or deprivation was also considered as an indirect effect of Cu on the PSII activity. Cu toxicity induces chlorosis of leaves. The question arising is whether to attribute this disturbance to a peroxidative breakdown of pigments (Sandmann and Böger 1980, Baszynski et al. 1988) or to specific inhibition of some steps of the biosynthetic pathways of chlorophyll and carotenoids. Some authors assume (see references in van Assche and Clijsters 1990) that Cu inhibits the synthesis of 5-amino-levulinic acid, as well as the protochlorophyllide reductase activity. Lidon and Henriques (1992) proposed that Fe and Mn deficiency triggered by excess Cu blocks synthesis of protochlorophyllide and phytoene, decreasing the contents of chlorophyll and carotenoids. Some authors had previously attributed decreased PSII electron transport to a reduction in photosynthetic pigments due to interference from terpenoid biosynthesis prior to the formation of C_{20} geranyl-geranyl-

pyrophosphate (Baszynski 1978, Henriques 1989, Droppa and Horvath 1990). However, the involvement of Cu in lipid metabolism and its interaction with these processes remain unclear at the moment. Their elucidation requires further experiments in vivo with Cu-poisoned and Cu-deprived plants.

Concluding remarks.

Since a Cu-free PSII preparation with high oxygen-evolving activity is available, reports on the association of Cu with PSII need to be reevaluated. Copper can no longer be considered a constituent of this photosystem. However, Cu might regulate PSII-mediated electron transport by maintaining an appropiate lipid and pigment composition.

Regarding the effect of Cu toxicity on PSII, the main inhibitory site appears to be located on the donor side, at or very close to the Tyr_z donor in the D1 protein. Secondary effects of Cu on the acceptor side near the Q_B site, cannot be excluded. The existence of a metal binding site nearby Tyr_z could have implications for the binding of other metals, e.g., Mn or Ca, on the donor side of PSII. Therefore, Cu treatment may become an useful instrument for functional studies of PSII and could contribute to clarifying the role of the different components of the electron transport chain.

Since most of the reviewed effects of Cu toxicity on PSII have been observed in vitro, further work with Cu-poisoned plants is needed to establish whether or not the physiological effects of Cu on photosynthesis are due to inhibition of electron donation in PSII.

Experiments in vivo are also necessary to clarify the interaction of Cu with lipid and

pigment synthesis. These could answer some intriguing questions about the indirect effect of Cu on the photochemical activities of the photo-systems due to a perturbation of photosynthetic membranes.

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