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Increase in the Quantum Yield of Photoinhibition Contributes to Copper Toxicity in Vivo[1]

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The effect of copper on photoinhibition of photosystem II in vivo was studied in bean (*Phaseolus vulgaris* L. cv Dufrix). The plants were grown hydroponically in the presence of various concentrations of Cu^{2+} ranging from the optimum 0.3 μM (control) to 15 μM. The copper concentration of leaves varied according to the nutrient medium from a control value of 13 mg kg^{-1} dry weight to 76 mg kg^{-1} dry weight. Leaf samples were illuminated in the presence and absence of lincomycin at different light intensities (500–1500 μmol photons m^{-2} s^{-1}). Lincomycin prevents the concurrent repair of photoinhibitory damage by blocking chloroplast protein synthesis. The photoinhibitory decrease in the light-saturated rate of O_2 evolution measured from thylakoids isolated from treated leaves correlated well with the decrease in the ratio of variable to maximum fluorescence measured from the leaf discs; therefore, the fluorescence ratio was used as a routine measurement of photoinhibition in vivo. Excess copper was found to affect the equilibrium between photoinhibition and repair, resulting in a decrease in the steady-state concentration of active photosystem II centers of illuminated leaves. This shift in equilibrium apparently resulted from an increase in the quantum yield of photoinhibition (Φ_{PI}) induced by excess copper. The kinetic pattern of photoinhibition and the independence of Φ_{PI} on photon flux density were not affected by excess copper. An increase in Φ_{PI} may contribute substantially to Cu^{2+} toxicity in certain plant species.

Cu^{2+} is an essential micronutrient but in excess is toxic for plants. It is a redox-active metal that functions as an enzyme activator and is an important part of prosthetic groups of many enzymes (for review, see Sandmann and Böger, 1983). Copper concentrations in healthy plant tissues range from 5 to 20 mg kg^{-1} dry weight. In Cu^{2+}-rich environments, accumulation of Cu^{2+} in plant tissues depends on the species and cultivar. Cu^{2+} seems to have several sites of action, which vary among plant species. Toxic concentrations of Cu^{2+} inhibit metabolic activity, which leads to suppressed growth and slow development. Most Cu^{2+} ions are immobilized to the cell walls of roots or of mycorrhizal fungi (Kahle, 1993).

When the tolerance mechanisms in the root zone become overloaded, Cu^{2+} is translocated by both the xylem and phloem up to the leaves. Excess Cu^{2+} may replace other metals in metalloproteins or may interact directly with SH groups of proteins (Uribe and Stark, 1982). Cu^{2+}-induced free-radical formation may also cause protein damage (for review, see Fernandes and Henriques, 1991; Weckx and Clijsters, 1996). High concentrations of Cu^{2+} may catalyze the formation of the hydroxyl radical from O_2 and H_2O_2. This Cu^{2+}-catalyzed Fenton-type reaction takes place mainly in chloroplasts (Sandmann and Böger, 1980). The hydroxyl radical may start the peroxidation of unsaturated membrane lipids and chlorophyll (Sandmann and Böger, 1980), and these inhibitory mechanisms might contribute to the observed inhibition of photosynthetic electron transport by excess Cu^{2+} (Clijsters and Van Assche, 1985).

The role of Cu^{2+} as an inhibitor of photosynthetic electron transport has been studied in vitro. Both the donor (Cedeno-Maldonado and Swader, 1972; Samuelsson and Öquist, 1980; Schröder et al., 1994) and acceptor (Mohanty et al., 1989; Yruela et al., 1992, 1993, 1996a, 1996b; Jegerschöld et al., 1995) sides of PSII have been proposed to be the most sensitive site for Cu^{2+} action. On the donor side, Cu^{2+} is thought to inhibit electron transport to P680, the primary donor of PSII (Schröder et al., 1994). On the acceptor side, Cu^{2+} interactions with the pheophytin-Q_A-Fe^{2+}-domain or Cu^{2+}-induced modifications in the amino acid or lipid structure close to the Q_A- and Q_B-binding sites have been suggested to cause the inhibition of electron transport (Jegerschöld et al., 1995; Yruela et al., 1996a, 1996b).

Celeno-Maldonado and Swader (1972) noticed that preincubation of chloroplasts in the light enhanced the Cu^{2+}-induced inhibition of electron transport, and that PSII was more susceptible to this kind of inhibition than was PSI. The hypothetical acceptor- and donor-side mechanisms of the light-induced inhibition of electron transport, photoinhibition, involve the same domains of attack as Cu^{2+}. Both acceptor- and donor-side photoinhibition trigger the D1 polypeptide of the PSII reaction center for degradation (for review, see Aro et al., 1993). The damaged D1 protein is degraded, and the recovery of PSII activity needs de novo synthesis of D1 protein. Photoinhibition occurs at all light intensities (Tyystjärvi and Aro, 1996); therefore, the cycle of PSII photoinhibition, which is followed by degradation,

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Abbreviations: F_m, maximal fluorescence; F_v, variable fluorescence; F_v/F_m, ratio of variable to maximal fluorescence; Φ_{PI}, quantum yield of photoinhibition; G, relationship between leaf copper concentration and the reaction rate constant of photoinhibition; I_a, photon flux density absorbed by PSII of the leaf; K_{DEG}, reaction rate constant of D1-protein degradation; K_{PI}, reaction rate constant of photoinhibition; Q_A and Q_B, primary and secondary electron-accepting plastoquinones of PSII; ρ_{PI}, effect of leaf copper concentration on K_{PI}.

and, finally, resynthesis of the D1 protein, runs constantly in plant cells in the light. If the photoinhibition-repair cycle is allowed to run for some time at a constant light intensity, equilibrium is reached. At equilibrium (steady state), all three reaction rates (photoinhibition, D1 degradation, and D1 synthesis) are equal. Healthy plants are often able to maintain a high steady-state concentration of active PSII under widely varying light intensities. Even if the concentration of active PSII is lowered by high light, the concentration of D1 protein tends to stay fairly constant (Cleland et al., 1990; Kettunen et al., 1991). In the bean (*Phaseolus vulgaris* L.) plants used in this study the steady-state D1 protein content remained almost constant even in the presence of excess Cu^{2+}.

The effect of Cu^{2+} on photoinhibition in vivo has been studied very little. Vavilin et al. (1995) suggest that excess Cu^{2+} may slow the PSII repair cycle in the green alga *Chlorella pyrenoidosa*, and Ouzounidou et al. (1997) suggest that Cu^{2+} inhibits adaptation to light in maize. In the current study we show that excess Cu^{2+} induces a large increase in the rate constant of photoinhibition in vivo in a higher plant.

MATERIALS AND METHODS

Bean (*Phaseolus vulgaris* L. cv Dufrix) seeds were sown in vermiculite. After 2 weeks the plantlets were moved to a hydroponic nutrient medium (Hoagland and Arnon, 1950) buffered with 2 mM Mes-KOH, pH 5.5. The nutrient medium was supplemented with nine different concentrations of $CuSO_4$, from 0.3 μM (control) to 15 μM. Nine plants were placed in each container with 5 L of nutrient solution, and the solution was changed twice a week. The plants were grown in a phytotron at 22°C in a 14-h light/2-h twilight/8-h dark rhythm for 2 weeks. The PPFD of the light phase was 250 μmol m^{-2} s^{-1}, which was reduced to 50 μmol m^{-2} s^{-1} during the twilight period. Three plants per treatment were used in each individual experiment, and each experiment was repeated at least three times.

Photoinhibitory Treatments and Fluorescence Induction Measurements

Leaves were harvested at the end of the dark period. The second pair of leaves was detached and the petioles were soaked for 3 h in lincomycin solution (1 g L^{-1} water) (before and during the measurement of K_{PI}) or in water alone (before and during the measurement of the equilibrium point of PSII photoinhibition and repair). The leaves were then illuminated in a temperature-controlled growth chamber at a PPFD of 500, 1000, or 1500 μmol m^{-2} s^{-1} for 4 to 5 h at 20°C with a 1200-W daylight metal halide arc lamp (color temperature 5600 K, Sylvania). Different experiments were done using plants from different cultivation batches.

During the experiment, six leaf discs per treatment were punched from the detached leaves every hour and dark adapted for 1 h between moist paper towels before fluorescence induction was measured with a fluorometer (PAM 101, Heinz Walz, Effeltrich, Germany) using fluorescence software (FIP, Q_A-Data, Turku, Finland). We also checked that the O_2 evolution activity did not change during the incubation period if measured from thylakoids isolated from the leaf discs (data not shown). Initial fluorescence was first measured under a dim-red measuring beam, and F_m was then induced with a 9000 μmol m^{-2} s^{-1} white-light pulse (KL-1500 illuminator, Schott, Mainz, Germany). The percentage of photoinhibitory decrease in PSII activity was calculated as $100 \times (F_v/F_m[\text{control}] - F_v/F_m[\text{treatment}])/F_v/F_m(\text{control})$. The relevance of the fluorescence measurements was checked by measuring O_2 evolution from thylakoids isolated from control and Cu^{2+}-treated leaves in the course of the photoinhibitory treatments.

Measurement of O_2 Evolution

O_2 evolution (water to 2,6-dichlorobenzoquinone) was measured with an O_2 electrode (Hansatech, King's Lynn, UK) from isolated thylakoids. Leaves were rapidly ground with a homogenizer (Ultra-Turrax, Janke and Kunkel, Staufen, Germany) in 50 mM Na-phosphate buffer, pH 7.4, 300 mM sorbitol, 5 mM $MgCl_2$, 1 mM EDTA, 1 M Gly-betaine, and 1% (w/v) BSA (added just before isolation). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged for 5 min at 1000*g*. The chloroplasts were resuspended in 5 mM sorbitol, 10 mM Hepes-KOH, pH 7.4, and 5 mM $MgCl_2$ to cause an osmotic shock. Thylakoids were then collected by centrifugation for 5 min at 2000*g* and resuspended in storage buffer solution containing 100 mM Suc, 25 mM Tris-HCl, pH 8.5, 5 mM NaCl, and 10 mM $MgCl_2$. PSII activity was measured in 40 mM Hepes-KOH, pH 7.6, 330 mM sorbitol, 5 mM NaCl, 5 mM $MgCl_2$, 1 M Gly-betaine, 1 mM $KHPO_4$, 5 mM NH_4Cl, and 0.25 mM 2,6-dichlorobenzoquinone. O_2 evolution was measured at 20°C in red light (plexiglass filter with a cutoff at 600 nm) using a slide projector as a light source. The PPFD was approximately 6500 μmol m^{-2} s^{-1} as measured by replacing the O_2 electrode cuvette with a light meter. It was tested by slightly reducing the light intensity so that the PPFD was high enough to saturate O_2 evolution in the samples. The chlorophyll concentration was 10 μg mL^{-1}.

Emission Spectra

Emission spectra of thylakoids isolated from control and Cu^{2+}-treated leaves were measured with a diode array fiber-optic spectrophotometer (S2000, Ocean Optics, Eerbeek, The Netherlands) exciting the samples at 435, 455, and 575 nm (10-nm half width) with a slide projector through an f/3.4 monochromator (Applied Photophysics, Surrey, UK). The wavelength resolution of the diode array was 3 nm. The sample (0.1 mL, 2.5 μg chlorophyll mL^{-1} storage buffer) was placed in an Eppendorf tube and frozen in liquid N_2 with the fiber-optic probe placed 1 mm from the surface. Self-absorption was carefully eliminated by diluting each sample until the ratio of the intensity of the emission band at 735 nm to that at 685 nm no longer changed with chlorophyll concentration.

Leaf Absorptivity

The absorptivity of control leaves and leaves of plants grown with 4 μM Cu^{2+} was measured with a 60-cm-diameter integrating sphere. The sphere was calibrated using a black standard, as described by Idle and Proctor (1983). Total absorptivity of the leaves was calculated by illuminating each leaf sample with the same lamp used in the photoinhibition experiments and measuring the PPFD inside the sphere with and without the leaf.

Relative Rate of D1-Protein Degradation

D1 protein was quantified by western blotting from thylakoids isolated from Cu^{2+}- and light-treated leaves. Samples were solubilized in Laemmli's solubilization buffer (Laemmli, 1970), except that 100 mM DTT was used instead of β-mercaptoethanol, and heated at 65°C for 5 min. The ratio of SDS to chlorophyll was approximately 800 μg μg^{-1}. The samples were loaded on the basis of their chlorophyll content (1 μg chlorophyll $well^{-1}$). Proteins were separated by SDS-PAGE using 14% acrylamide gels that contained 4 M urea. The stacking gel contained 4% acrylamide and 4 M urea. Proteins were transferred to PVDF membrane (Millipore). D1-specific antibody (Research Genetics, Huntsville, AL), raised against amino acids 234 to 242 of *Synechocystis* 6803 D1 protein, was used as primary antibody. Goat anti-rabbit alkaline phosphatase was used as the secondary antibody (Caltag Laboratories, Burlingame, CA), and the immunodetection of the D1 protein was performed using a chemiluminescence kit (Bio-Rad). Immunoblots were quantified with a charge-coupled device camera and software (MCID, St. Catharine's, Ontario, Canada).

Analysis of Basic Elements

The concentrations of basic elements (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) in leaves, roots, and stems were measured with a plasma emission spectrophotometer (ICP-AES, Applied Research Laboratories, Lausanne, Switzerland). The number of copper ions per PSII reaction center was calculated by assuming that the antenna size of PSII was 210 and that of PSI was 230 chlorophyll units per reaction center, and that the ratio of PSI to PSII was 1.2.

Chlorophyll Determination

Chlorophyll was measured according to the method of Porra et al. (1989).

Mathematical Modeling

Photoinhibition treatments in the presence of lincomycin were used to measure K_{PI}. K_{PI} was extracted by fitting the photoinhibitory decrease in F_v/F_m to a first-order equation (Tyystjärvi et al., 1994). G was established with the following equation:

$$K_{PI} = \Phi_{PI} \times I_a \times G([Cu]) \quad (1)$$

The numerical value of Φ_{PI} was obtained by multiplying the estimated number of PSII centers per unit area in a nonphotoinhibited leaf by K_{PI}, and dividing the result by the number of quanta absorbed by PSII in unit time and unit area. The estimate of the number of PSII centers in the unit area was based on the same assumptions used in calculating the number of Cu^{2+} ions per PSII center, and 39% of the quanta incident on the leaf were assumed to end up in PSII in control leaves; this number was based on a leaf absorptivity of 75% (Table I). The same assumptions could be used for the Cu^{2+}-treated leaves, despite their lower chlorophyll content, because the number of quanta absorbed per unit time was found to be roughly proportional to the chlorophyll content per unit area (Table I).

We assumed that the D1 protein of the photoinhibited PSII center was degraded in a first-order reaction (Tyystjärvi et al., 1994). The following treatment applies only to

Table I. *Dark-adapted F_v/F_m ratios, light-saturated rates of O_2 evolution, chlorophyll contents, and leaf absorptivities (400–700 nm)*

The light-saturated rate of O_2 evolution was measured from thylakoids isolated from bean plants grown for 2 weeks in the presence of 0.3 to 15 μM Cu^{2+}; the other parameters were determined from the leaves. The F_v/F_m and O_2 evolution measurements were done on 5 to 10 different cultivation batches, as described, and the chlorophyll (Chl) and absoptivity data are based on measurements from one cultivation batch.

Cu^{2+} Concentration of Growth Medium	F_v/F_m	O_2 Evolution	Chl Content	Light Absorptivity
μM		$\mu mol\ mg^{-1}\ Chl\ h^{-1}$	$g\ m^{-2}$	
0.3	0.837 (n = 9)	439 (n = 6)	0.16	0.75
1	0.850 (n = 6)	*[a]	*	*
2	0.836 (n = 6)	*	0.16	*
3	0.839 (n = 7)	*	*	*
4	0.809 (n = 10)	402 (n = 5)	0.15	0.59
6	0.751 (n = 6)	*	*	*
8	0.744 (n = 6)	*	*	*
12	0.750 (n = 6)	*	*	*
15	0.738 (n = 6)	*	0.11	*

[a] *, Not determined.

the lincomycin-treated leaves with no synthesis of the D1 protein; however, we assume that the K_{DEG} value is the same if protein synthesis is allowed. Equations 2 and 3 describe photoinhibition and degradation of the D1 protein in the presence of lincomycin.

$$dB/dt = K_{PI}A - K_{DEG}B \quad (2)$$

$$dC/dt = K_{DEG}B \quad (3)$$

In Equations 2 and 3, A, B, and C are the concentrations of active, inhibited, and D1-depleted PSII centers, respectively, and t is time. At the beginning of each photoinhibition experiment, A was set to F_v/F_m of the nonphotoinhibited sample divided by F_v/F_m of the nonphotoinhibited control samples grown without excess Cu^{2+}, B was set to $1 - A$, and C was set to 0. The smallest initial value of A was 0.88. Table I lists the F_v/F_m values used. The model was optimized assuming that Cu^{2+} does not affect K_{DEG}. Thereafter, the optimization was done by assuming a linear effect of leaf Cu^{2+} content on K_{DEG}. ModelMaker software (Cherwell Scientific Publishing, Oxford, UK) was used for the optimization.

RESULTS

Visible Damage and Copper Concentration of the Leaves

Both root and shoot growth of the bean plants decreased with increasing copper concentration. Chlorosis and necrotic spots increased in the leaves, and browning of the roots increased with increasing Cu^{2+} concentration in the growth medium. After the 2-week Cu^{2+} treatment, visible damage was most obvious in the youngest leaves, whereas the primary leaves and the second pair of leaves showed less visible symptoms of Cu^{2+} stress. The visible symptoms were observed if the Cu^{2+} concentration in the growth medium was 2 μM or more.

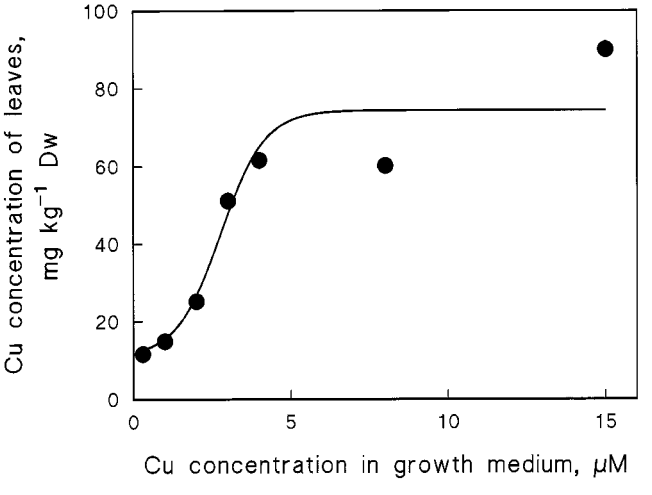

Figure 1. Copper concentration in the second pair of bean leaves after 2 weeks of hydroponic growth at different concentrations of Cu_2SO_4. The curve represents the best fit (minimum = 11 mg kg^{-1}; maximum = 74 mg kg^{-1}; k = 1.4 μM^{-1}; and $\times 50$ = 2.8 μM) to a logistic sigmoid relationship, $[Cu^{2+}](\text{leaf}) = \text{minimum} + (\text{maximum} - \text{minimum})/(1 + e^{-k([Cu^{2+}]\text{medium} - \times 50)})$, between the leaf copper concentration and the concentration of Cu^{2+} in the medium. Dw, Dry weight.

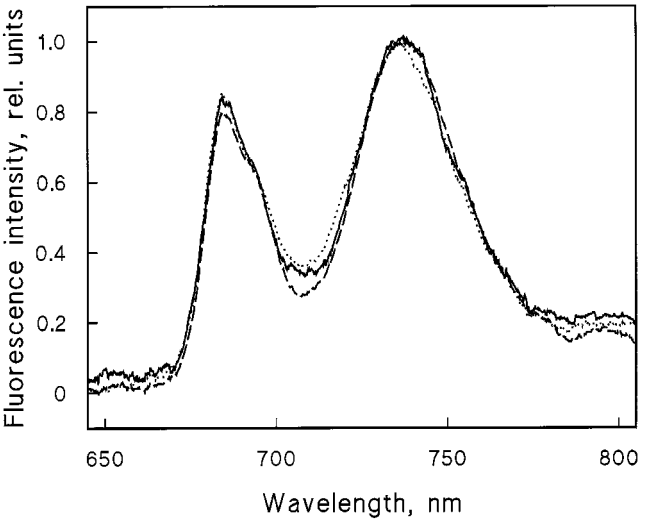

Figure 2. Emission spectra of thylakoids isolated from control (solid line) plants and plants grown with 4 μM (dashed line) or 15 μM Cu^{2+} (dotted line) measured with a diode-array fiber-optic spectrophotometer at 77 K. The sample volume was 0.1 mL, the chlorophyll concentration was 2.5 μg/mL, and the excitation was 455 nm.

Analysis of basic elements showed that the copper concentration of the second pair of leaves was a sigmoidal function of the Cu^{2+} concentration of the growth medium. The uptake saturated at 4 to 6 μM. The best fit of the experimental data to a logistic sigmoid curve (Fig. 1; see the figure legend for the equation) was later used as an estimate of the copper content.

The chlorophyll content of the leaves treated with the highest Cu^{2+}concentration was 69% of control (Table I), but the characteristics of the photosystems were similar. Emission spectra measured with 455 nm excitation (Fig. 2) showed that the Cu^{2+} treatments did not affect the ratio of PSII to PSI; the same result was obtained by 435 and 575 nm excitation (data not shown). Cu^{2+} treatment (4 μM) induced a similar decrease in the F_v/F_m ratios of the leaves and in the light-saturated rate of O_2 evolution (expressed per milligram of chlorophyll; Table I). The unchanged ratio of active PSII per unit of chlorophyll indicates that the Cu^{2+} treatment did not induce major changes in the antenna sizes of the photosystems. Based on the measurements of the chlorophyll content of control and Cu^{2+}-treated leaves (Table I), and assuming that each PSII center is associated with 210 and each PSI center with 230 chlorophyll molecules and that the ratio of PSII to PSI is 1.2, the copper concentration of 13 to 76 mg kg^{-1} dry weight equals 10 to 110 copper ions per PSII reaction center. For measurements of antenna sizes and photosystem stoichiometry in higher plants, see Melis (1996).

The highest copper concentrations were measured in the roots, where they were 10 to 20 times higher than in the second pair of leaves. The copper concentration per kilogram dry weight had a tendency to decrease with the age of the leaf (data not shown). The concentrations of Fe and

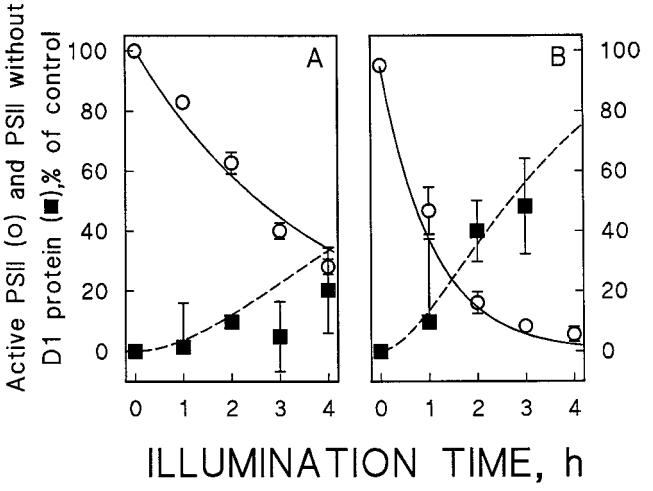

Figure 3. Photoinhibition and D1 degradation in the leaves of bean plants grown at two different Cu^{2+} concentrations. The leaves of control plants (A) and plants grown with 4 μM Cu^{2+} (B) were illuminated at a PPFD of 1000 μmol photons m^{-2} s^{-1} in the presence of lincomycin. The lines show the best fit to the model obtained using data collected from all photoinhibition treatments in the presence of lincomycin. The solid lines indicate the concentration of active PSII centers, measured as 100 × F_v/F_m(sample)÷F_v/F_m(control) (○). The dashed lines represent the number of D1-depleted PSII centers (■); the K_{DEG} value is 0.3 h^{-1} in both A and B. Each data point represents the mean of three independent experiments; three leaves from three different plants were used for each experiment, and different experiments were done with plants from different cultivation batches. The error bars indicate SE and are shown only if larger than the symbol.

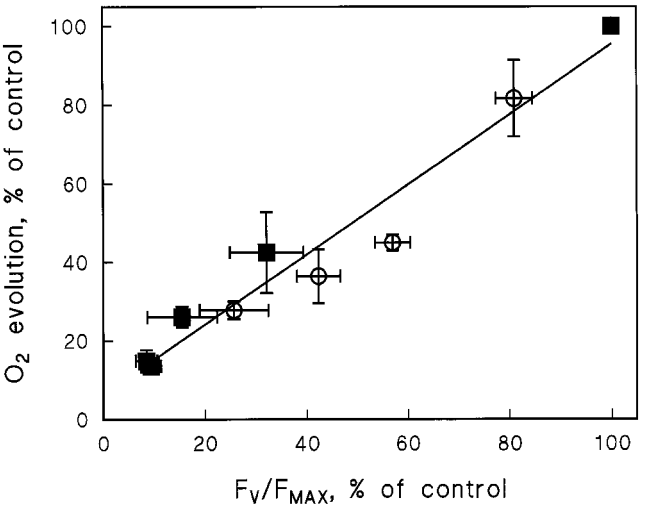

Figure 4. Correlation of O_2 evolution activity with the F_v/F_m ratio (r^2 = 0.95). O_2 evolution was measured from thylakoids isolated after photoinhibition treatments (1000 μmol photons m^{-2} s^{-1}) of control leaves (○) and leaves grown with excess (4 μM) Cu^{2+} (■). F_v/F_m was measured from leaf discs. Each data point represents the mean of three independent experiments. The error bars indicate SE and are shown only if larger than the symbol.

Mn in the leaves correlated negatively with the concentration of copper. No differences in the concentrations of other basic elements (Ca, K, Mg, P, S, and Zn) were observed (data not shown).

Effect of Cu^{2+} on Photoinhibition: The Damaging Reaction

Photoinhibition measured in the presence of lincomycin followed the first-order equation in both the presence and absence of excess Cu^{2+}, as deduced from the decrease in the F_v/F_m ratio (Fig. 3, solid lines). The light-saturated rate of O_2 evolution in thylakoids isolated from treated leaves showed a good linear correlation with the F_v/F_m ratio during photoinhibition treatments (Fig. 4).

The first-order nature of photoinhibition allowed us to extract K_{PI}. By measuring in three different light intensities (Fig. 5), it was verified that excess Cu^{2+} did not affect the basic linearity of K_{PI} as a function of light intensity (Tyystjärvi and Aro, 1996). This linearity allowed us to approximate Φ_{PI}, which is the probability that a PSII center becomes photoinhibited upon absorbing a photon.

To elucidate an expression for the function G (Eq. 1), leaves with different copper concentrations were illuminated in the presence of lincomycin, and the K_{PI} values were compared. Excess Cu^{2+} induced an increase in K_{PI}, and this effect depended linearly on the copper concentration of the leaves (Fig. 6). Function G is thus reduced to a constant factor ρ_{PI} as follows:

$$K_{PI} = \Phi_{PI} \times I_a \times (1 + \rho_{PI} \times [Cu]_{excess}), \quad (4)$$

where ρ_{PI} is a constant, $[Cu]_{excess}$ is obtained by subtracting the control value 13 mg kg^{-1} dry weight from the leaf copper concentration, and I_a is the PPFD absorbed by PSII of the leaf. The excess concentration was used in Equation 4 instead of total copper concentration of the leaf because suboptimal Cu^{2+} concentrations were not tested. The

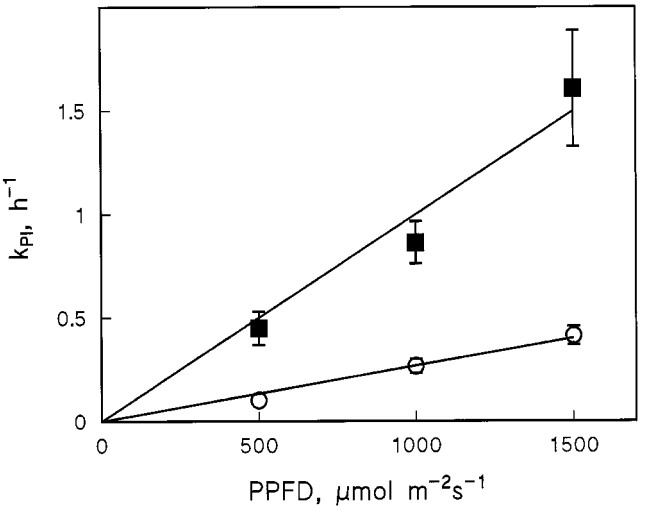

Figure 5. K_{PI} in control (○) and 4 μM Cu^{2+}-treated (■) bean leaves measured at three different light intensities. Each K_{PI} value was determined from a first-order fit to the results of a similar 4-h photoinhibition experiment as shown in Figure 2. Error bars indicate SE and are shown only if larger than the symbol.

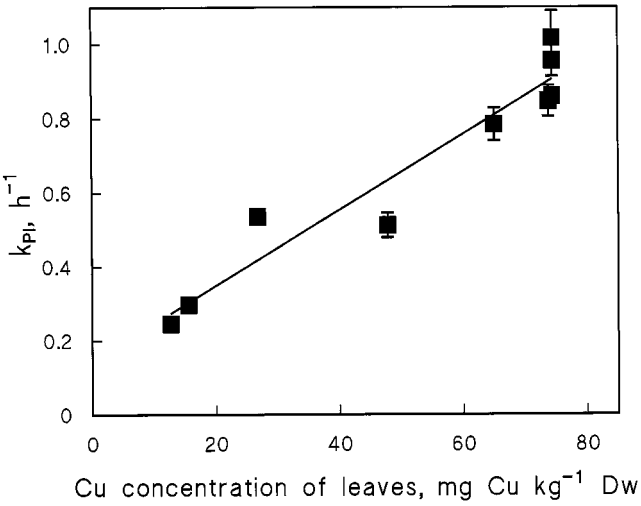

Figure 6. K_{PI} measured at a PPFD of 1000 μmol photons m^{-2} s^{-1}, plotted against the copper concentration of the bean leaves. The copper concentrations of the leaves were read from the curve of Figure 1. The K_{PI} values were determined from first-order kinetic analysis of similar experiments as those shown in Figure 2. Each data point represents a K_{PI} value derived from the curve, which includes F_v/F_m data from three independent experiments. The solid line shows the best linear fit. Error bars indicate SE and are shown only if larger than the symbol. DW, Dry weight.

optimized value of ρ_{PI} is 0.047 kg mg^{-1} dry weight (Table II), which results in a 3.8-fold increase in K_{PI} when going from the control Cu^{2+} concentration to 15 μM Cu^{2+} in the growth medium (76 mg kg^{-1} dry weight in the leaves; Fig. 6).

Effect of Excess Copper on the Repair of Photoinhibited PSII

When bean leaves were illuminated at a PPFD of 1000 μmol photons m^{-2} s^{-1}, the repair cycle approached an equilibrium after 3 h (Fig. 7). Excess copper lowered the equilibrium level of active PSII, and the extent of the lowering was dependent on the copper concentration of the leaves. At the optimum copper concentration 65% of PSII centers remained active at the equilibrium, whereas only 25% remained active at the highest copper concentration (Fig. 7, ○, solid lines). The same tendency was seen in the

Table II. *Optimized values of the parameters of the model of photoinhibition and D1 protein degradation*

Parameter[a]	Explanation	Value	SE
Φ_{PI}	Quantum yield of photoinhibition	1.7×10^{-7}	
K_{DEG}	Rate constant of degradation of damaged D1 protein	0.3 h^{-1}	0.02
ρ_{PI}	Effect of leaf copper concentration on K_{PI}	0.047 kg mg^{-1}	0.02

[a] See Equations 1 to 4 for the exact definitions of the parameters.

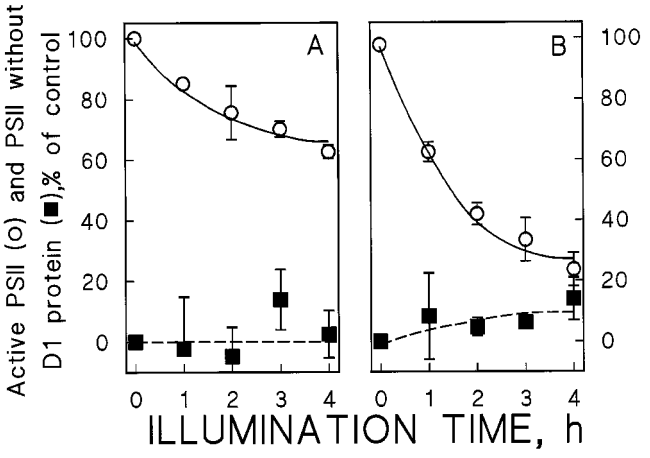

Figure 7. The effect of excess copper on the equilibrium between photoinhibition and repair. The leaves of control plants (A) and plants grown with 4 μM Cu^{2+} (B) were illuminated at 1000 μmol photons m^{-2} s^{-1} in the absence of lincomycin. The Cu^{2+} concentrations of the growth media were 0.3 μM (A) and 4 μM (B). The solid lines indicate the concentration of active PSII centers measured with F_v/F_m (○), the dashed lines represent the amount of D1-depleted PSII centers (■). Each data point represents three independent experiments, three leaves from three different plants were used for each experiment, and different experiments were done with plants from different cultivation batches. The error bars indicate SE and are shown only if larger than the symbol.

F_v/F_m ratios and in the O_2 evolution rates measured before the light treatment (Table I), confirming that a slight displacement of the equilibrium occurs also during growth of the plants.

To find out whether the decrease in the equilibrium level is entirely caused by the copper-induced increase in Φ_{PI}, we measured D1-protein degradation during similar 4-h photoinhibitory treatments in the presence and absence of lincomycin. We have earlier shown that degradation of the damaged D1 protein also obeys first-order kinetics when assayed in vitro or in the presence of a chloroplast protein synthesis inhibitor in vivo (Tyystjärvi et al., 1994). When the model was optimized, allowing excess copper to affect the value of K_{DEG}, the magnitude of the effect was found to be too small to be significant in the limits of accuracy of the D1-protein determinations. The data therefore suggest that after the photoinhibitory loss of PSII activity, the damaged D1 protein was degraded with similar kinetics in control plants and plants grown with excess Cu^{2+} (Fig. 3). If the degradation of the damaged D1 protein is independent of protein synthesis, as suggested by results in cyanobacteria (Kanervo et al., 1993), then the rate-limiting step of the PSII repair cycle in our bean plants was the degradation of the damaged protein, with a half-time of 2 h (K_{DEG} value of 0.3 h^{-1}; Table II). Figure 7 shows that once the D1 protein is degraded, it is very rapidly replaced by a new copy and, therefore, little loss of D1 protein can be seen in the absence of lincomycin.

DISCUSSION

We examined the effect of excess copper on PSII photoinhibition and the repair cycle in vivo using copper con-

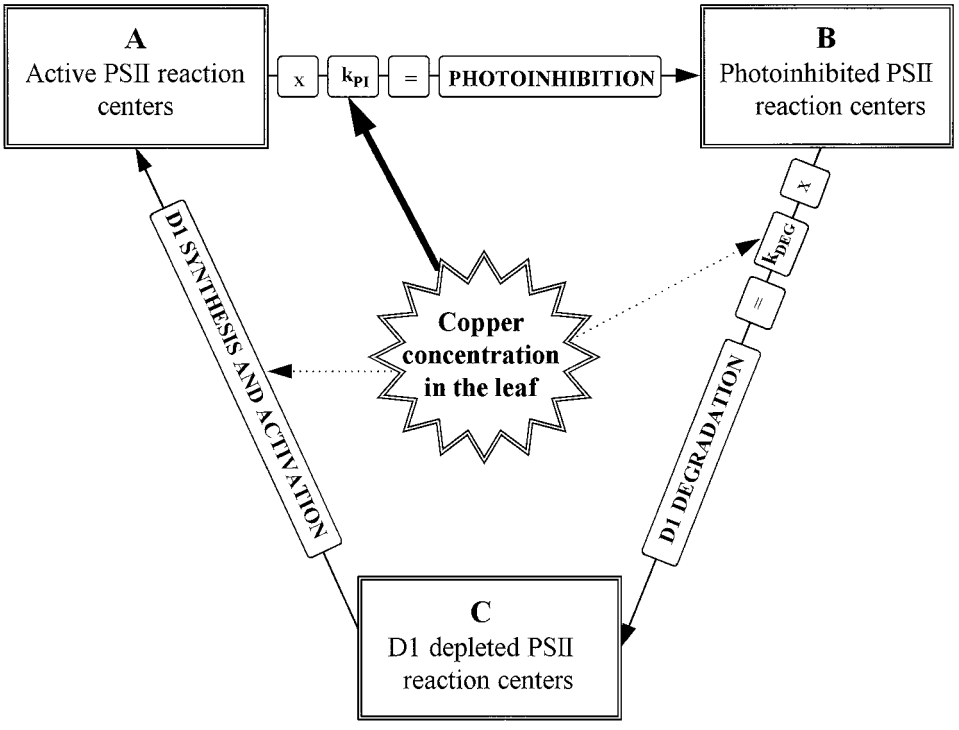

Figure 8. Model of the effect of copper on the repair cycle of photoinhibition. In the presence of lincomycin the behavior of the model (without copper effects) is determined by Equations 2 and 3.

centrations found in plants growing in polluted areas. Although copper accumulated mainly in roots, the copper level of leaves also increased, causing, in addition to enhancement of photoinhibition, chlorosis and necrotic spots in plants growing in the highest concentrations of Cu^{2+} used.

The cycle of photoinhibition and repair of PSII consists of a light-induced loss of active PSII reaction centers, which are subsequently repaired via enzymatic degradation and resynthesis of the D1 protein (for reviews, see Prasil et al., 1992; Aro et al., 1993). The damaging step is a first-order reaction and its quantum yield is independent of light intensity (Tyystjärvi and Aro, 1996). These key kinetic features of the damaging reaction were unaffected by toxic concentrations of copper (Figs. 3 and 5), indicating that the effect of excess copper was simply to speed up the same photoinhibition reaction that occurs in the absence of Cu^{2+} stress. In control leaves, the relationship between K_{PI} and PPFD was virtually the same in this study as it was when measured in pumpkin in our earlier study (Tyystjärvi and Aro, 1996). PSII is thus equally vulnerable to light-induced damage in bean and pumpkin leaves. Slow degradation of the damaged D1 protein (Fig. 3), reflected by the low value of K_{DEG} (Table II), seems to be a frequently occurring feature of higher plants grown under low light (Tyystjärvi et al., 1992; Schnettger et al., 1994). It is possible that this slowness is related to phosphorylation of the D1 protein (Aro et al., 1992).

Simple enhancement of photoinhibition by excess Cu^{2+} may be restricted to physiologically relevant concentrations. In vitro experiments suggest that illumination in the presence of very high Cu^{2+} concentrations induces a light-dependent inactivation mechanism different from that functioning in the absence of excess Cu^{2+} (Jegerschöld et al., 1995; Yruela et al., 1996b). In the present study, leaf Cu^{2+} concentrations were 10 to 110 Cu^{2+} ions per PSII reaction center, whereas concentrations far above 250 Cu^{2+} ions per PSII reaction center have been used in vitro (Jegerschöld et al., 1995; Yruela et al., 1996b).

Copper might cause an increase in Φ_{PI} in two ways: (a) the metal ion may directly participate in the damaging reaction, e.g. by enhancing the production of hydroxyl radicals (Sandmann and Böger, 1980; Yruela et al., 1996b) or chlorophyll triplets (Sandmann and Böger, 1980); or (b) the toxic metal may block a protective reaction or induce a change in the structure of photosynthetic membranes or proteins (DeVos et al., 1989; Weckx et al., 1996), thereby inducing an increase in Φ_{PI}, without taking part in the damaging reaction itself. Indeed, Ouzounidou et al. (1997) suggest that excess Cu^{2+} inhibits the process of light adaptation in intact leaves. The data presented here cannot unequivocally distinguish between these two possibilities. However, the protective mechanisms are expected to be induced upon saturation of photosynthesis (Demmig-Adams and Adams, 1996), whereas Φ_{PI} was independent of light intensity both in the presence and in the absence of excess copper. This finding suggests that copper directly enhances the damaging reaction.

Elucidation of the mechanism by which copper speeds up photoinhibition is hampered by the fact that the mechanism of photoinhibition is unknown in vivo. Two main hypotheses on the molecular mechanism of photoinhibi-

tion have been put forward. The acceptor-side mechanism (Vass et al., 1992) depends on accumulation of double-reduced Q_A, and would therefore be enhanced by a Cu^{2+}-induced inhibition of electron transfer from Q_A to Q_B (Yruela et al., 1991, 1992). However, the acceptor-side mechanism probably is not the mechanism of photoinhibition in vivo (Tyystjärvi and Aro, 1996). On the other hand, Cu^{2+}-induced enhancement of donor-side inhibition would be conceivable, since excess Cu^{2+} inhibits electron donation from water to PSII (Schröder et al., 1994; Jegerschöld et al., 1995). An argument against this mechanism is that donor-side inhibition proceeds in the absence of O_2, and O_2 has been shown to affect photoinhibition in vivo (Van Wijk and Krause, 1992; Leitsch et al., 1994). Donor-side-induced inhibition of PSII was seen when a huge Cu^{2+} concentration was used in vitro (Jegerschöld et al., 1995).

The effect of copper on Φ_{PI} was linear in the range of 10 to 110 copper ions per PSII, suggesting that the copper effect is caused by free copper ions or binding of copper to one binding site in PSII. The copper concentrations of chloroplasts may also differ from the overall concentrations in the leaves.

In an earlier in vivo study, only a minor excess of Cu^{2+} in the growth medium of the green alga *Chlorella pyrenoidosa* was found to inhibit the recovery from photoinhibition without affecting the rate of the damaging step (Vavilin et al., 1995). Our results show that in a higher plant excess copper has a major effect on the damaging reaction, but we also addressed the question of whether copper has additional, physiologically important effects on the repair mechanism.

Figure 8 summarizes our findings on the effect of excess copper on photoinhibition in vivo. Our results suggest that the copper-induced decrease in the equilibrium level of active PSII is mainly caused by an increase in Φ_{PI}.

The repair of PSII after photoinhibition starts with degradation of the damaged D1 protein. The resolution of the D1 data does not allow strong conclusions, but it is clear that the D1 protein of the photoinhibited PSII centers was degraded in both Cu^{2+}-treated and control plants (Fig. 3), and the data do not suggest any retardation of the degradation reaction by excess copper. This result is in contrast with the in vitro results of Yruela et al. (1996b), which essentially showed that the Cu^{2+}-induced enhancement of photoinhibition was not accompanied by enhanced degradation of the D1 protein.

The effects on D1-protein synthesis are not directly accessible from our data because no good mathematical model is available and because PSII heterogeneity may further complicate the cycle (Melis, 1991) from the simple model shown in Figure 8. A feedback mechanism in D1 synthesis of cyanobacteria has been suggested (Tyystjärvi et al., 1996), and in higher plants, D1-protein synthesis is adjusted to match the rate of the photoinhibition-induced degradation (Kettunen et al., 1997). The constancy of the level of D1 protein in photoinhibition experiments both in the presence and absence of excess copper (Fig. 7) suggests that neither the synthesis rate of the D1 protein nor the feedback mechanism is significantly affected by the Cu^{2+} treatments.

Plants can be divided into three categories according to their reaction to excess Cu^{2+} (Baker, 1981): an average plant such as bean is of the "indicator" type and takes up metal ions at a linear rate according to the amount of metal ions in the soil; "excluders" are able to keep the metal ions outside of the roots (DeVos et al., 1991); and a few grasses are Cu^{2+}-tolerant "accumulators" or metallophytes (Ernst et al., 1992). Trees usually accumulate heavy metals in their roots (Kahle, 1993), and indeed, our preliminary experiments on birch have shown that under hydroponic growth conditions, translocation of excess Cu^{2+} to leaves is very slow, and Cu^{2+}-induced damage is mainly targeted to roots (E. Pätsikkä, E. Tyystjärvi, and E.-M. Aro, unpublished results).

It is obvious that no single mechanism can explain everything about copper toxicity in plants. Photoinhibition is a universal cost factor decreasing the overall yield of photosynthesis, and both in vitro (Mohanty et al., 1989; Yruela et al., 1996b) and in vivo evidence (this study) suggests that excess copper speeds up photoinhibition. Like some other environmental constraints such as low temperature, excess copper displaces the equilibrium between photoinhibition and recovery reactions toward a more inhibited state, enhancing the adverse effects of light. Earlier results from a green alga and maize (Vavilin et al., 1995; Ouzounidou et al., 1997) suggest that such displacement may occur in different ways in different photosynthetic organisms. This behavior suggests that photoinhibition may contribute significantly to copper toxicity in indicator-type species.

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