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In situ detection of heavy metal substituted chlorophylls in water plants

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Abstract

The *in vivo* substitution of magnesium, the central atom of chlorophyll, by heavy metals (mercury, copper, cadmium, nickel, zinc, lead) leads to a breakdown in photosynthesis and is an important damage mechanism in heavy metal-stressed plants. In this study, a number of methods are presented for the efficient *in situ* detection of this substitution (i.e. in whole plants or in chloroplasts). While macroscopic observations point to the formation of heavy metal chlorophylls at higher concentrations, fluorescence microscopy enables the detection of this reaction at very low substitution rates. Therefore, the course of the reaction can be followed by continuously measuring the fluorescence of whole plants. Furthermore absorbance spectroscopy of whole cells or isolated chloroplasts also enables the *in situ* detection of heavy metal chlorophylls. These methods provide practicable approaches in detecting the formation of these compounds *in situ*, avoiding artefacts that might occur using extraction methods based on polar solvents. In addition to the new methods for *in situ* detection, an extreme heterogeneity in the reaction of cells in the same tissue upon heavy metal stress was observed: while some cells are already disintegrating, others still show normal fluorescence and photosynthetic activity. Measurements of fluorescence kinetics gave a further hint that in high light intensity a substitution of Mg by heavy metals might take place specifically in PS II reaction centres.

Abbreviations: chl – chlorophyll; DCMU – 3-(3,4 Dichlorophenyl)-1,1-Dimethylurea; F_0 – basic ('dark') fluorescence; F_m – maximum fluorescence; F_v – variable fluorescence; $F_{steadystate}$ – steady state fluorescence, i.e. after fluorescence induction; hm^{2+} – heavy $metal^{2+}$; hms – heavy metal-substituted; Mg-substitution – substitution of the natural central ion of chlorophyll, Mg^{2+}, by heavy metals

Introduction

The toxicity of heavy metals to plant metabolism has received extensive research interest for several decades and has been widely reviewed (e.g. Fernandes and Henriques 1991; Markert 1993). The substitution of the Mg^{2+} ion in the chlorophyll molecule by certain toxic heavy metals such as Cu, Zn, Cd or Hg has been shown to occur during heavy metal stress in higher plants, resulting in a breakdown of photosynthesis (Küpper et al. 1996). For example, results obtained from cyanobacteria (Kowalewska and Hoffmann 1989) and unicellular chlorophytes such as *Chlorella* (De Filippis 1979; Gross et al. 1970) and the photosymbionts of lichens (Puckett 1976) support this interpretation.

Most hms-chls are unsuitable for photosynthesis: their much lower *in vitro* fluorescence quantum yields compared to Mg-chl (Watanabe and Kobayashi 1988) indicate a rather unstable first excitation state which relaxes thermally; the resonance energy transfer from the antenna pigment complexes to the re-

action centres in the thylakoids (which depends on the same excitation states that cause chl fluorescence (e.g. Karukstis 1991) is therefore not possible. In addition, Mg-chl *a* has the highest capacity to release electrons from the singlet excited state, compared to all hms-chls (Watanabe et al. 1985). Hence, the formation of these compounds in a significant proportion of the photosynthetic pigments during heavy metal stress will lead to a complete breakdown of photosynthesis (Küpper et al. 1996). Interestingly, a photosynthetically active Zn-bacteriochlorophyll has been reported recently from *Acidiphilium rubrum*, an acidophilic phototrophic bacterium (Wakao et al. 1996). In this special case, however, the use of Zn-bacteriochlorophyll appears reasonable in maintaining a stable photosynthetic pigment under highly acidic conditions (Mg-chl would be converted to pheophytin). This effect obviously overcompensates the lower photosynthetic quantum yield of the Zn complex, which is about 75% lower compared to Mg-chl (Watanabe and Kobayashi 1988). Nevertheless, Zn-chl formed in a photosynthetic system consisting of Mg-chl (such as in higher plants) cannot be functional because the blue-shifted absorbance and fluorescence maxima of Zn-chl will destroy the spectral overlap of fluorescence/absorbance bands in the antenna complexes which is necessary for excitation transfer towards the reaction centres.

In addition to the photochemical effects, membranes (Sandmann and Böger 1980), plastocyanin (Kimimura and Katoh 1972), mineral metabolism (e.g. Pirson 1958; Sims 1986) and various enzymes (e.g. Clijsters and Van Assche 1985) are also affected by excess concentrations of heavy metal ions in the plant cell. Also such effects contribute to the inhibition of photosynthesis by heavy metals.

Due to possible artefacts that may occur during extraction of the photosynthetic pigments (Küpper et al. 1996), application of non-invasive techniques in detecting the formation of hms-chls *in situ* is desirable.

Basically, changes in both absorption spectra and fluorescence characteristics can be used for *in situ* detection of hms-chls. For example, absorption spectroscopy methods rely on the blue shifts occurring during Mg-substitution (Watanabe and Kobayashi 1988). Detection of hms-chls by measurement and observation of steady-state fluorescence is based on the lower fluorescence quantum yields of these substances compared to Mg-chl (Watanabe and Kobayashi 1988).

In this study, non-invasive spectroscopic, fluorimetric and fluorescence microscopic techniques are described that enable *in situ* detection of hms-chl formation. A chlorococcoid green alga (*Scenedesmus quadricauda*) and some higher plants (*Elodea canadensis*, *Callitriche stagnalis*, *Ceratophyllum demersum*) were chosen to test the general validity of results.

Materials and methods

Plant material and growth conditions

The following species were used: *Elodea canadensis* Michx., *Callitriche stagnalis* Scop., *Ceratophyllum demersum* L. and *Scenedesmus quadricauda* (strain Greifswald 15). *E. canadensis*, *Callitriche stagnalis* and *Ceratophyllum demersum* were collected from noncontaminated ponds. Macrophytes (higher plants) were grown in nutrient solution (Gaudet 1963) between 2–5 days to acclimatise the plants to the experimental conditions applied subsequently. *Scenedesmus quadricauda* was kindly provided by Dr Ivan Šetlik (Microbiological Institute of the Czech Academy of Sciences, Třeboň). Plants were stressed for 1–2 weeks with soluble $CuSO_4$ $CdCl_2$ or $ZnSO_4$, (Merck p.a.). In the case of *S. quadricauda*, heavy metals (hm^{2+}) were added to the nutrient solution in only one pulse (in our experiments: $c(hm^{2+}) = 1 \times 10^{-6}$ to 1×10^{-3} mol l^{-1}). The aqueous concentration decreased due to uptake of hms into organic tissue, simulating a single entry of a heavy metal into an aquatic habitat.

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Figure 1. Comparison of symptoms in case of sun and shade reaction: (A) *Callitriche stagnalis*: unstressed specimens on the left, specimens stressed with copper in shade in the middle, specimens stressed (for 2 weeks) with a single addition of 50 μmol l^{-1} copper in sunlight on the right. (B) *Ceratophyllum demersum*: *left*: unstressed specimen, *right*: specimens stressed (for 2 weeks) with a constant concentration of 2 μmol l^{-1} copper in shade. (C) Microscopic photograph of *Elodea canadensis* cells stressed (for 2 weeks) with a single addition of 50 μmol l^{-1} cadmium in sunlight. (D) *Elodea canadensis*: unstressed specimens on the left, specimens stressed (for one week) with a constant copper concentration of 2 μmol l^{-1} in sunlight on the right. (E) *Elodea canadensis*: unstressed specimens on the right, specimens stressed (for one week) with a constant copper concentration of 2 μmol l^{-1} in shade on the left.

Figure 1.

By contrast, constant concentrations (in experiments with higher plants: 5×10^{-8} to 2×10^{-4} mol l^{-1}) simulating a permanent contamination were achieved by exchanging solutions continuously. Experiments with macrophytes were carried out as a comparison between natural shade (up to 2 W m^{-2}) and natural sunlight (up to 150 W m^{-2}). In experiments with *Scenedesmus*, a comparison between low-intensity (up to 5 W m^{-2}) and high-intensity (up to 200 W m^{-2}) artificial (tungsten) light with 16 h/8 h light/dark-rhythm was used instead.

Isolation of chloroplasts and pigments

For the isolation of chloroplasts from stressed and unstressed plants, the procedure described by Walker (1988) was applied. Pigments were extracted as described previously (Küpper et al. 1996).

Analytical methods

Macro- and microscopic photographs were taken of all *in vivo* experiments to document changes in colour, growth, tissue- and cell-structure. For fluorescence microscopy, blue excitation light (400–500 nm) was used combined with a green or red cut-off filter. Photos were taken with Kodak Ektachrome EPJ 320 T (320 ISO / 26°) and Kodak Ektachrome 160 T (160 ISO / 24°) films (both for tungsten light).

Spectra of intact leaves, intact *S. quadricauda* specimens and isolated chloroplasts were measured with photometers specially adapted for recording spectra of turbid samples: In order to minimise the loss of information due to light scattering, the detector has to be positioned directly behind the cuvettes (head-on). Additionally, all samples were extracted and examined spectroscopically for a quantitative assay of hms-chls, as described in Küpper et al. (1996). Both absorbance and fluorescence spectra were recorded using UV/VIS photometers linked to a computer for subsequent calculations.

In vivo steady state fluorescence (after fluorescence induction) was measured with blue (400–500 nm) excitation light and red (>600 nm) cut-off-filter. Kinetics of fluorescence induction were measured using a Waltz PAM modulated fluorimeter with 80 W m^{-2} actinic (tungsten) light.

Results

Upon substitution of Mg in the chl molecules by heavy metals (subsequently termed 'Mg-substitution'), photosynthesis of stressed plants underwent an abrupt cessation (Figure 1; Küpper et al. 1996).

The intensity of light irradiance has striking consequences for the symptoms resulting from heavy metal stress:

Reaction in weak light ('Shade Reaction')

The total chlorophyll content of heavy metal-stressed plants decreased only slightly under shady conditions. At least in the case of Cu-stressed specimens, most of this slight decrease in absorbance is caused by the low absorptivity of Cu-chl. As a result of Mg-substitution, chloroplasts of damaged plants exhibited a colour change, corresponding to the colour of the hms-chl formed, e.g. in the case of copper blue-green (Figures 1a, b, e). Because of the high stability of Cu- and Zn-chl, plants stressed with copper or zinc in shade remained green and often appeared vital (Figures 1a, b, e) even when they were already dead and disintegrating.

Fluorescence measurements

Since Cu-chl shows no fluorescence, formation of Cu-chl *in vivo* should lead to a decrease of observable fluorescence. Therefore, fluorescence measurements on plants treated with Cu^{2+} were carried out. The ratios F_0/F_v, F_0/F_m and $F_{steadystate}/F_m$ decreased compared to the control, F_v/F_m increased (Table 1). The decrease in steady state fluorescence indicates the formation of (non-fluorescent) Cu-chl, which could be traced by absorbance spectroscopy (see below). However, this fluorescence decrease was much greater than the rate of Mg-substitution (Küpper et al. 1996). We used this phenomenon for another method of *in situ*-detection of Cu- and Zn-chl: fluorescence microscopy.

Fluorescence microscopy

Because of the rather similar colour of Zn-chl compared to Mg-chl, formation of Zn-chl *in vivo* is hardly visible in transmittant light. But there is already a strong decrease in fluorescence in damaged cells (Figures 2, 3) because the *in vitro* fluorescence quantum yield (indicative of the stability of the singlet excited state, compare introduction) of Zn-chl is about one fourth compared to Mg-chl (Watanabe and Kobayashi 1988). Additionally, the emission maximum of Zn-chl

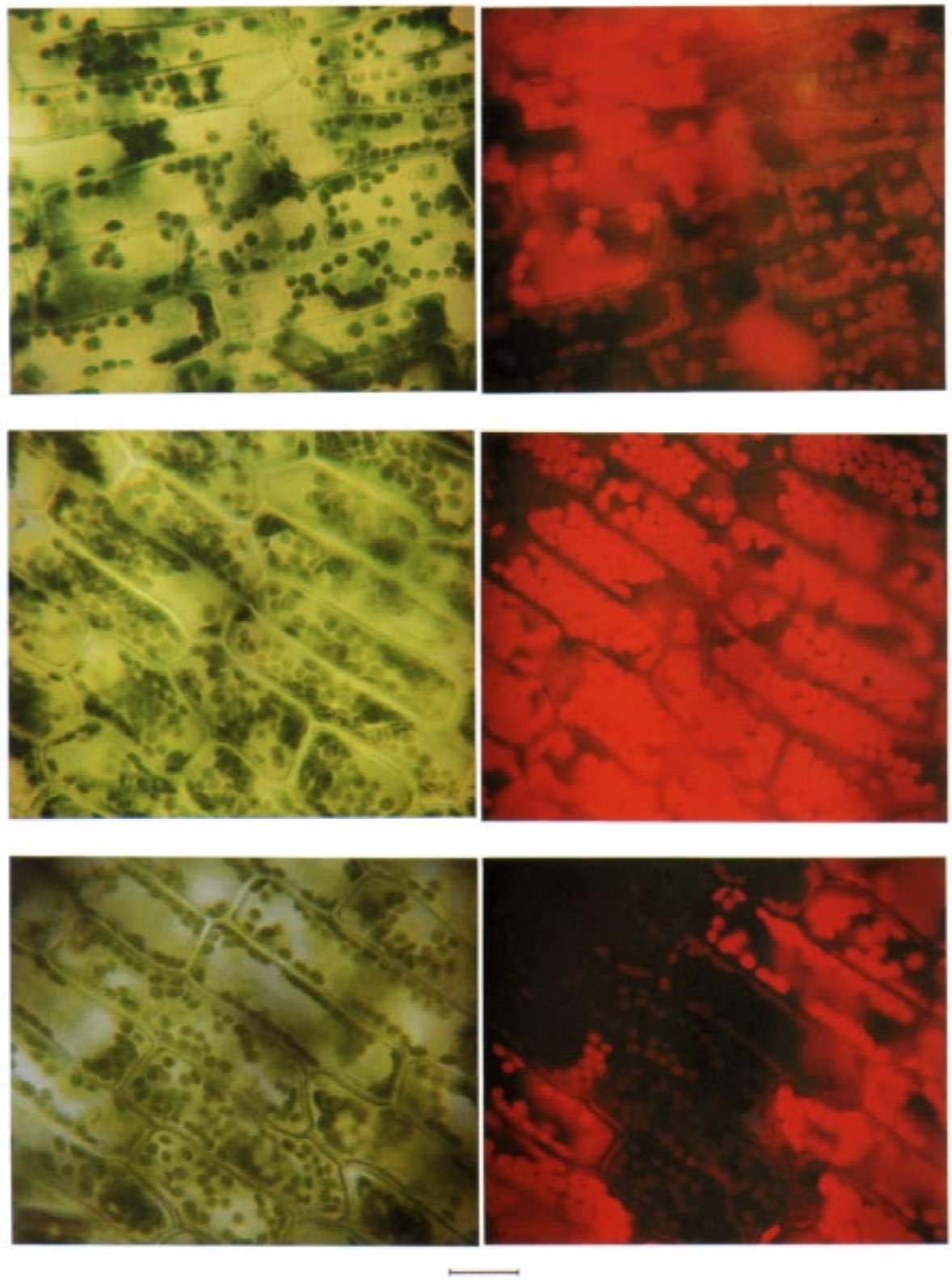

Figure 2. Comparison of transmittant and fluorescence microscopic photographs of *Elodea canadensis* stressed in shade (the bar represents 20 μm): *left*: transmission; *right*: fluorescence with red cut-off filter; *up*: stressed for one week with a constant concentration of 100 μmol l^{-1} zinc for one week; *middle*: unstressed; *down*: stressed for one week with a constant concentration of 1 μmol l^{-1} copper for one week.

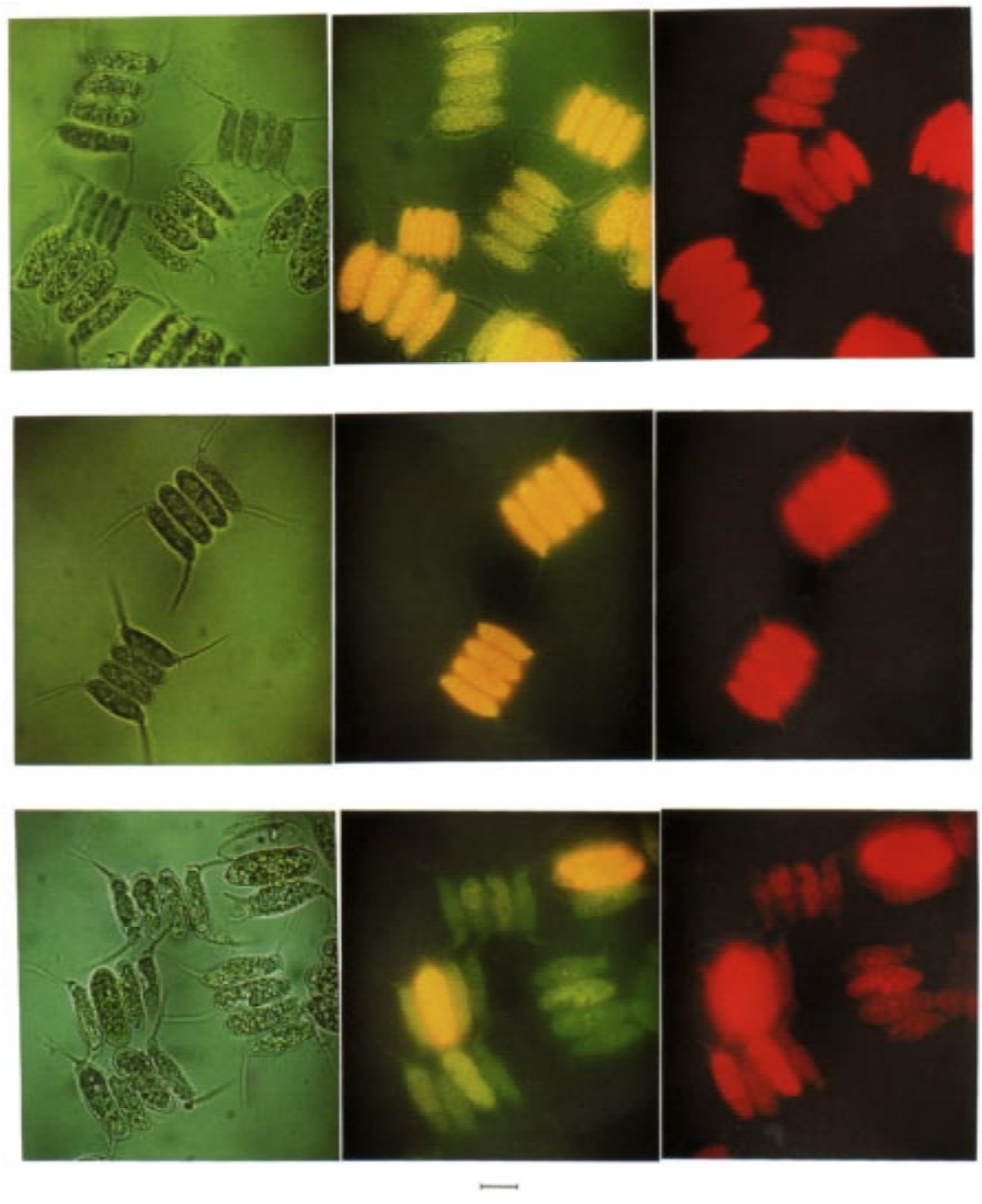

Figure 3. Comparison of transmittant and fluorescence microscopic photographs of *Scenedesmus quadricauda* stressed in shade (the bar represents 10 μm): *left*: transmission; *middle*: fluorescence with green cut-off filter; *right*: fluorescence with red cut-off filter; *up*: stressed with a single addition of 100 μmol l^{-1} zinc for 4 days; *middle*: unstressed; *down*: stressed with a single addition of 10 μmol l^{-1} copper for 4 days.

is slightly (ca. 5 nm) blue-shifted. Also in the case of Cu-chl the detection with fluorescence microscopy is much easier than with transmittant light and also easier than with absorbance spectroscopy. Cells that died because of copper stress exhibit hardly any red fluorescence, since Cu-chl is non-fluorescent. In contrast, Zn^{2+}-treated plants still exhibit a weak red fluorescence (Figures 2, 3) even at high Zn^{2+} concentrations, which is a result of the weak fluorescence of Zn-chl.

Remarkably, cells of stressed plants react quite heterogeneously upon heavy metal stress (Figures 2, 3): while some cells are still intact even in highly stressed specimens and produce a measurable photosynthetic activity, other cells are completely dead, and some of them are already disintegrating.

In vivo *spectroscopy*

In vivo spectroscopy of entire specimens or isolated chloroplasts allows *in situ*-detection of hms-chlorophylls as well: The formation of hms-chlorophylls in this case becomes visible as a shift of the red absorbance maximum (Figures 4a, b). Treatment with phosphoric acid of isolated chloroplasts from unstressed *E. canadensis* specimens resulted in a shift of the red absorbance maximum from about 682 nm to about 695 nm, due to conversion of Mg-chl to pheophytin (Figure 4a). This peak at 695 nm *(in vivo)* could be shown to belong to pheophytin by extracting the pigments after the treatment with phosphoric acid and examining the extract again spectroscopically. Treatment of *S. quadricauda* cells with phosphoric acid resulted in a shift of the red absorbance maximum from 678 nm to 685 nm due to the same effect (Figure 4b). In the case of Cu- and Zn- stressed *E. canadensis* and *S. quadricauda* this shift could not be observed, which once more demonstrates the formation of the more stable Cu- or Zn-chlorophylls (Figures 4a, b).

Reaction in strong light ('Sun Reaction')

Under strong light, heavy metal-treated plants bleached almost completely due to chlorophyll-decay even in the case of Cu^{2+} (Figures 1a, d). A spectroscopic analysis of cyclohexane extracts from copper-stressed plants reveals that only a very small proportion (< 2%) of the total chl is converted to Cu-chl. In the case of Cd^{2+}, chlorophyll is completely degraded (Figure 1c) because Cd-chl is highly unstable (Küpper et al. 1996).

These findings show that in sunlight only a small proportion of the chlorophyll molecules (<2%) is accessible to substitution by heavy metal ions. Otherwise, Mg-substitution by heavy metals would inevitably take place, so that stressed plants would remain green, at least in the case of Cu^{2+} and Ni^{2+}. Both Cu-chl and Ni-chl are so stable that they do not bleach, even after weeks of exposure to direct sunlight, neither in plants which were damaged in shade nor in solutions. Therefore, if heavy metals that form **stable** chl complexes (e.g. Cu^{2+}, Zn^{2+}) are used, the pigment which remains after bleaching of Cu- or Zn-stressed plants is mainly hms-chl (Figure 4c).

Because of the low proportion of substituted chl, detection of Mg-substitution in case of heavy metal stress in high light intensity is rather difficult: the detection of hms-chls by measuring the absorbance of an intact plant or an extract is only possible after bleaching of the Mg-chlorophyll. Finally, measuring the fluorescence changes of plants in this case is not suitable at all: since almost all the chlorophyll remains unsubstituted, only the bleaching of the Mg-chlorophyll causes a fluorescence decrease, which is much slower than the inhibition of photosynthesis. However, the ratio of dark to variable fluorescence (F_0/F_v) is much greater in plants damaged with copper in high light intensity compared to the control and also compared to plants damaged in shade. In contrast, the ratio F_v/F_m is much smaller than in control specimens and plants stressed in low light intensity (Table 1).

Discussion

Non-invasive techniques based on absorption spectroscopy, fluorimetry and epifluorescence microscopy provide promising approaches to further study the damage inflicted to plant metabolism by the insertion of Cu, Zn and other toxic heavy metals in the place of Mg in photosynthetically active chl. In particular, both *in vivo* absorption and fluorescence spectroscopy as well as fluorescence microscopy have proven powerful means to achieve this aim.

The shade reaction

Under these conditions, practically all the chl can be converted to hms-chls (Küpper et al. 1996).

The different sensitivity of PS II/PS I has been described repeatedly before (Atal et al. 1991; Clijsters and Van Assche 1985; Gross et al. 1970). Combined with the finding that the fluorescence decrease

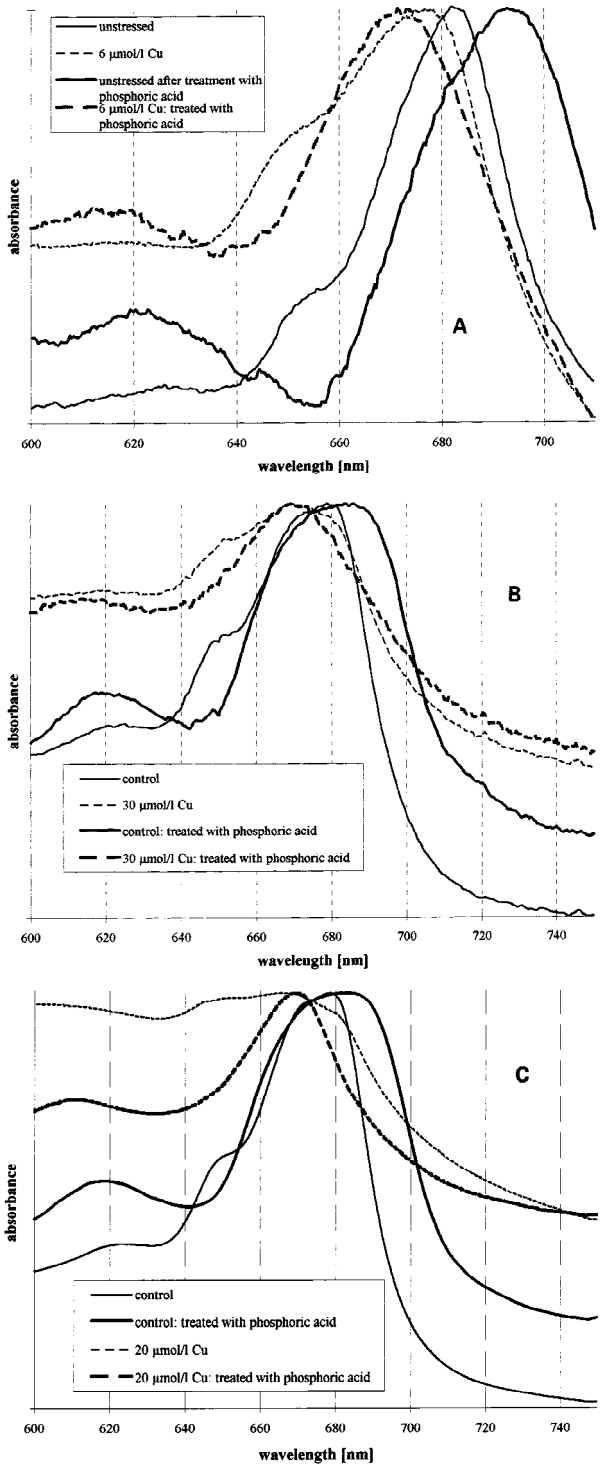

Figure 4. In vivo absorbance spectra: for better comparison, the red absorbance maxima are scaled to a common height. (A) Absorbance spectra of suspended *E. canadensis* chloroplasts isolated after 12 days of constant copper stress in shade (< 2W m^{-2}). (B) Absorbance spectra of suspended *Scenedesmus quadricauda* cells after 4 days of copper stress caused by a single addition of 30 μmol l^{-1} Cu^{2+} in low intensity (5W m^{-2}) tungsten light. (C) Absorbance spectra of suspended *Scenedesmus quadricauda* cells after 4 days of copper stress caused by a single addition of 20 μmol l^{-1} Cu^{2+} in high intensity (150W m^{-2}) tungsten light. Note that also these spectra are normalized; the chlorophyll content of cells after damage in high light intensity is greatly decreased (see 'Results').

Table 1. Scenedesmus quadricauda stressed with copper in low (<5 W m^{-2}, 'shade') and high (up to 200 W m^{-2}, 'sun') light intensity: parameters of fluorescence kinetics

	F_0/F_v	F_0/F_m	F_v/F_m	$F_{steadystate}/F_m$
Control, shade	3.8	0.79	0.21	0.85
10 μm Cu, shade	2.0	0.67	0.33	0.74
Control, sun	2.8	0.74	0.26	0.79
10 μm Cu, sun	7.3	0.88	0.12	0.83

Abbreviations: F_0 = basic ('dark') fluorescence F_v = variable fluorescence F_m = maximum fluorescence $F_{steadystate}$ = steady state fluorescence, i.e. after fluorescence induction.

is greater than the rate of substitution and the fact that most of the fluorescence is caused by PS II, this points at a Mg-substitution specifically in chlorophylls associated with PS II.

Many authors have published spectra that demonstrate the formation of hms-chls in heavy metal-stressed plants. However, most of these studies refer to lichens and microscopic algae (De Filippis 1979; Gross et al. 1970; Kowalewska and Hoffmann 1989; Puckett 1976; Samuelsson and Öquist 1980) rather than higher plants (Küpper et al. 1996). With the only exception of the latter study, none of them considers the formation of these compounds in the context of a damage mechanism and all of them are based on the extraction of photosynthetic pigments after the exposure of the plants to toxic heavy metals. As discussed before, this approach has to be taken with a certain caution due to artefacts that may be formed during the extraction process under certain circumstances. The application of non-invasive techniques to study this process *in situ* appears desirable in order to provide further insight into the process of damage.

In vivo *spectroscopy*

In vivo spectroscopy turned out to be an effective tool for the detection of hms-chl *in situ*. The blue-shift of the red absorbance maximum was also used by Wakao et al. (1996) for the detection of Zn-bacteriochlorophyll in *Acidiphilium rubrum*. The application of phosphoric acid, which leads to a conversion of Mg-chlorophyll to pheophytin, makes the detection of stable hms-chls (such as Cu-chl, Zn-chl, Ni-chl) much easier: it enlarges the difference between the absorbance maxima of substituted and unsubstituted pigment from at most 10 nm to up to 20 nm. However, non-invasive detection of hms-chl formation by absorption spectroscopy of whole chloroplasts or tissues compared to detection in solutions requires special adaptation of the apparatus, and sometimes the detected effects are rather subtle. In contrast to this, observations of *in vivo* chl fluorescence of affected plants are easy to carry out and reveal striking differences.

Fluorescence measurements

Chlorophyll fluorescence has been widely established as a tool in plant stress physiology in the past two decades (e.g. Krause and Weis 1984; Lichtenthaler and Rinderle 1988). Although a decrease of both variable (F_v; e.g. Bernier et al. 1993) and steady-state chl fluorescence has been commonly observed as a consequence of heavy metal stress (e.g. Atal et al. 1991; Bernier et al. 1993; El-Sheekh 1993; Krupa et al. 1993; Lanaras et al. 1993; Lidon et al. 1993; Ouzounidou 1993; Sgardelis et al. 1994; Tripathy and Mohanty 1981), few authors have previously considered the possibility of using the decrease of steady state chl fluorescence as a parameter for the formation of Cu-chl (Küpper et al. 1996; Wu and Lorenzen 1984). Also the changes of the other fluorescence kinetics parameters (F_0, F_m) measured in copper-stressed plants can be explained by a decrease in PS II antenna size caused by formation of non-fluorescent, inactive Cu-chl.

Fluorescence microscopy

Fluorimetric methods also include the use of epifluorescence microscopy which provides an interesting link between photometric and cytological studies concerning the effects of heavy metals on plant metabolism. This clearly has the advantage of well-adapted, suitable apparatus being widely available, because this technique has been applied in the biological sciences for several decades, in particular in plant science and cell biology. For hydrobiological applications, epifluorescence microscopy is a wide-

spread tool to detect chlorophyll and phycobiliproteins in phytoplankton (e.g. Furuya 1990; Hall 1991; Tsuji et al. 1986). In a comparable case to the one discussed in this work, Lehnen et al. (1990) used fluorescence microscopy for a histochemical detection of protoporphyrin IX as a result of plant treatment with acifluorfen. Another application of fluorescence microscopy for the intracellular detection of fluorescent porphyrins has been proposed recently by Georgiou et al. (1994). Unlike those many other applications of epifluorescence microscopy in other fields of plant science, this method has never been applied so far for the *in vivo* detection of hms-chls. However this approach appears straightforward: Using blue excitation light, chloroplasts with unaffected Mg-chl will exhibit bright red fluorescence, whereas plastids with hms-chls will appear low- or non-fluorescent. To our knowledge, this paper reports this application of blue light epifluorescence microscopy for the first time.

The observed heterogeneity of damage in cells of a single tissue might have several reasons. It might represent a random distribution of heavy metal resistance, demonstrating phenotypic variability of cells within a tissue. But it might also be a hint towards an active stress response reaction of the plants: In order to minimise damage caused by inevitable heavy metal uptake, plants might sequester these heavy metals in a few cells. These cells used for dumping heavy metals would die, but this would ensure the survival of the remaining cells, which were kept at low intracellular heavy metal concentrations.

The sun reaction

The low substitution rate restricts any detection of the substitution that is based on the measurement of the total chlorophyll content to the time after bleaching of the other pigments. However, if substitution in sunlight occurs only in chl molecules bound to a few specific proteins (e.g. reaction centres of PS II (Atal et al. 1991; Li and Miles 1975)), an isolation of single pigment–protein complexes using green gel systems (Allen and Staehelin 1991) might enable a detection of the substitution at an earlier stage. Such a site-specific Mg-substitution in the PS II reaction centres is also in agreement with the high F_0/F_v (and low F_v/F_m) compared to the control and shade reaction: Formation of Cu-chl in the core of PS II (e.g. in the 'special pair' or pheophytin) would lead to inhibition of PS II, so that energy harvested by the antenna would be emitted as fluorescence. This would be an alternative hypothesis to the assumption that heavy metals directly interact with polypeptide chains in PS II. In contrast to the hypothesis of inhibition by interaction with polypeptides, our hypothesis might also explain the different sensitivity of PS I and PS II: In contrast to PS I, PS II contains pheophytin, which is (at least in isolated form) much more susceptible to complexation of heavy metals than the Mg-chl of the 'special pair'.

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