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Transformation of protochlorophyllide to chlorophyllide in wheat under heavy metal stress.

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Introduction

Among various heavy-metal pollutants the most phytotoxic agents are Cd, Cr, Fe, Pb, Cu and Zn. Ions of these metals present in the environment can be the reason of a reduced plant growth, permanent damage of cells, tissues and organs and they can eventually lead to death. Heavy metals affect biosynthesis of photosynthetic pigments and assembly of the photosynthetic apparatus, which frequently results in chlorosis.

There is growing evidence on the influence of heavy metals on particular steps of chlorophyll (Chl) biosynthesis. However, relatively little is known about their effect on protochlorophyllide (Pchlide) to chlorophyllide (Chlide) photoreduction. In angiosperms growing in darkness, biosynthesis of Chl is halted at the step of Pchlide formation (Sundqvist and Dahlin 1997; Schoefs 1999). The reduction of Pchlide to Chlide is triggered by light. This reaction is catalysed by the nuclear-encoded enzyme NADPH:protochlorophyllide oxidoreductase (POR). In etiolated plants, Pchlide is accumulated in the etioplast inner membranes (EPIM), as a spectrally inhomogeneous pool. Four spectral forms of Pchlide were found having absorption maxima at 630, 645, 650, 670 nm (Böddi et al. 1992). Only the Pchlide associated with POR, having the 77 K fluorescence maximum at 655-657 nm can be reduced upon light. The photoinactive Pchlide has a fluorescence maximum at 633 nm.

Up to now, only the influence of cadmium on the spectral properties of Pchlide accumulated in etiolated plants was reported (Böddi et al. 1995). The effect was observed as the decrease of the 77 K fluorescence intensity of photoactive Pchlide and the increase of the band of photoinactive Pchlide. Similar results were found by Stobart et al. (1985) for 77 K absorption spectra. On the contrary, Horváth et al. (1996) found that neither Pchlide accumulation nor its transformation to Chlide were inhibited by cadmium.

In the present work the influence of Cd^{2+}, Cr^{6+}, Fe^{3+}, Cu^{2+} on spectral properties of accumulated Pchlide and Pchlide to Chlide photoreduction was investigated.

Materials and methods

Experiments described in this work were performed on EPIM isolated from wheat (*Triticum aestivum*) seedlings that were grown in darkness for 7 days at 23°C on Hoagland medium. After removing the top 1-cm parts, the following 5-cm pieces of the leaves were used for EPIM isolation using the method of Ouazzani Chahdi et al. (1998). The isolation medium was 25 mM HEPES-NaOH buffer (pH 7.8) containing 0.4 M sorbitol, 1 mM $MgCl_2$ and 1 mM EDTA. Harvesting of etiolated leaves, EPIM isolation and all experiments were performed at 4°C under dim green light.

Fluorescence emission spectra were measured at 77 K using PERKIN-ELMER LS-50B spectrofluorometer. The excitation was at 440 nm.

Results

In order to characterize the influence of heavy metal ions on the stability of photoactive Pchlide as well as the yield of its photoreduction, the 77 K fluorescence emission spectra were measured during the incubation of EPIM suspension with different metal ions. The following ions and concentrations were used in the experiments: Cd^{2+} (10 μM, 1 mM, 10 mM), Cu^{2+} (25 μM, 100 μM, 1 mM), Fe^{3+} (10 μM, 1 mM, 10 mM), Cr^{6+} (1 mM, 10 mM). All experiments were repeated tree times.

A typical spectrum of an EPIM suspension has the main peak at 655 nm emitted from the photoactive Pchlide and the second peak at 633 nm related to photoinactive Pchlide. During incubation of EPIM with heavy metal ions used in our study the decrease of the 655-nm peak and the increase of that at 633 nm were observed. Kinetics of these changes varied depending on the kind of metal applied. An example of spectral changes for EPIM treated with Cr^{6+} is shown in Fig 1.

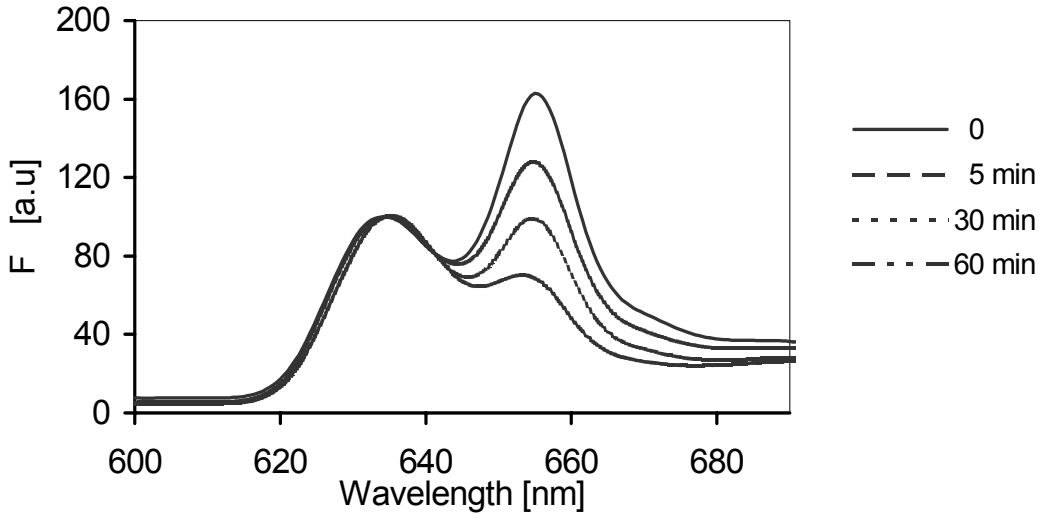

Fig.1. Changes in the fluorescence spectra of EPIM suspension treated with 10 mM Cr^{6+}. All spectra were normalized at 633 nm. The spectrum of untreated sample (control) is the same as for time 0.

From the obtained spectra, fluorescence intensities were measured at 633 (F633) and 655 nm (F655) and a parameter F655/F633 was calculated. The obtained values of F655/F633 for all metal ions are presented in the Table 1.

Table 1. Relative ratio of fluorescence intensities at 655 and 633 nm (F655/F633) calculated from fluorescence emission spectra measured during incubation of EPIM with metal ions. F655/F633 for the untreated sample at the beginning of experiment was treated as 1. (* - non-measurable)

Metal	Conc.	Time [min]				
	[mM]	0	5	15	30	60
Cd^{2+}	1	0,53	0,49	0,40	*	*
	10	0,54	0,36	*	*	*
Cu^{2+}	1	1,13	1,02	1,07	1,03	1,00
Fe^{3+}	10	0,96	0,93	0,84	0,79	0,69
Cr^{6+}	10	0,95	0,83	0,66	0,58	0,48

The solution of Cd^{2+} (10 mM) led to the fastest decrease of the F655/F633 parameter. After 5 min the 655-nm peak disappeared and the ratio could not be calculated. For 1 mM Cd^{2+} the F655/F633 ratio could only be calculated for 15 min. The solution of Fe^{3+} and Cr^{6+} were less toxic for the stability of the photoactive Pchlide. However, in both cases the decrease of the F655/F633 parameter was observed during the first hour of incubation. In the same time period, the value of F655/F633 for Cu^{2+} solution was almost constant. The photoactive Pchlide was more stable under Fe^{3+} than under Cr^{6+} treatment. In the case of Fe^{3+}, the decrease of F655/F633 of about 50 % was observed approximately 4.5 h after Fe^{3+} addition. What is more, a hipsochromic shift (2-3 nm) of the 655-nm maximum was observed after 15-min treatment for Cd^{2+}, after 1 h for Cr^{6+} and after 4 h for Fe^{3+}. However, as long as the peak with the maximum at 653-655 nm was distinguishable, its photoreducibility was observed.

For lower metal concentrations, the decrease of F655/F633 value was also observed, but, the changes were less distinct in comparison with the untreated sample (data not shown). Thus, only the highest metal concentrations were used in further experiments.

To investigate the role of NADPH in the protection of the photoactive Pchlide against metals, the above experiments were repeated for EPIM incubated with metals in the presence of NADPH (0.2 mM). The NADPH molecule is a hydrogen donor in the reaction of Pchlide to Chlide photoreduction (Begley and Young, 1989) and probably stabilizes the EPIM structure (Ryberg and Sundqvist, 1988). Presence of NADPH in the incubation medium resulted in a slower decrease of the 655-nm peak. However, even if the equimolar quantities of NADPH and cadmium (10 mM) were added to the EPIM suspension, the decrease of 655-nm peak with concomitant increase of the 633-nm peak were still observed (Fig. 2).

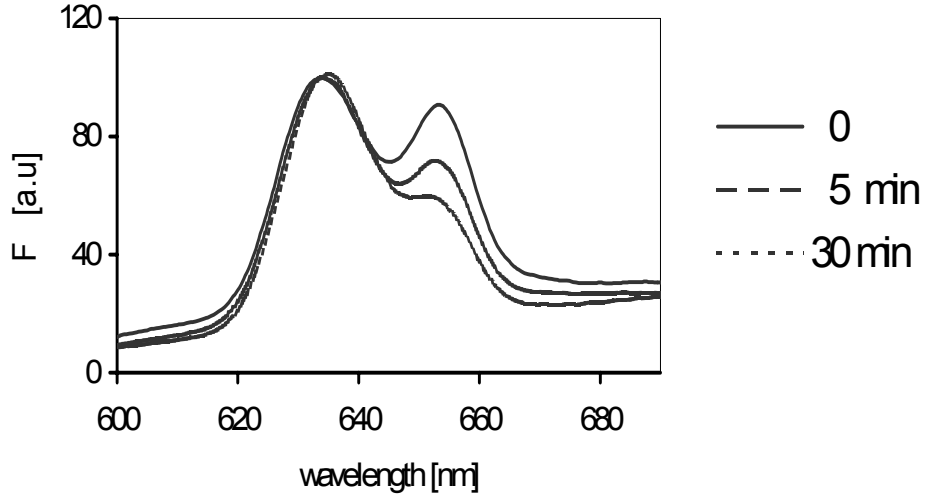

Fig.2. Changes in the fluorescence spectra of EPIM suspension treated with 10 mM Cd^{2+} in the presence of 10 mM NADP. All spectra were normalized at 633 nm.

In another experiments, other reducing agents such as NADH (0.2 mM) and Cys (0.2 mM) instead of NADPH were added to the EPIM suspension, however, a protective effect on photoactive Pchlide stability was not found. Also the presence of 1 mM AMP or 0.2 mM D-gluocose-6-phosphate were ineffective in this respect.

Discussion

Pchlide is one of chlorophyll precursors. Thus, the quantity of Chl accumulated in plant cells depends on the rate of the synthesis and photoconversion of Pchlide. It was shown in the present investigation that heavy metals like Cd, Fe and Cr influence the spectral properties of Pchlide accumulated in EPIM isolated from etiolated wheat. In particular, the stability of the photoactive Pchlide form is lower in the presence of these metal ions. When transformed to the form having fluorescence emission maximum at 633 nm Pchlide became photoinactive.

Distinct changes in the Pchlide spectrum were observed for the following concentrations of metals: 10 mM of Cd^{2+}, Fe^{3+}, Cr^{6+} and 1 mM of Cd^{2+}. Copper did not show any effect even at 10 mM concentration. Comparing the obtained results, it can be concluded that Cd^{2+} has the strongest effect on the stability of the photoactive Pchlide. For 10 mM Cd^{2+}, the decay of F655 was already observed after 5 min. Just after Cd^{2+} addition to EPIM suspension the value of F655/F633 decreased by about 50 % in relation to the untreated sample. Addition of the 10 mM Fe^{3+} and Cr^{+6} resulted in the decrease of this ratio from 96% to 69% and from 95% to 48%, respectively, during 1 h, whereas the maximal decrease of the F655/F633 ratio for the control sample was less than 15 % during 1 h.

In conclusion, it can be stated that:

- Cd^{2+}, Cr^{6+}, Fe^{3+} cause the decrease of the amount of photoactive Pchlide when added to wheat EPIM suspension,
- addition of NADPH can lower the toxic effect of metal on the stability of the photoactive Pchlide;
- unlikely to NADPH, NADH, Cys, AMP, D-glucose-6-phosphate do not protect the stability of the photoactive Pchlide against heavy metals,
- toxicity of the investigated metals on the Pchlide to Chlide photoreduction can be ordered as Cd > Cr > Fe, no effect was observed for Cu ions.

The mechanism of interaction of the investigated metal ions with photoactive Pchlide form has not been revealed yet and needs further investigations.

Acknowledgements

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