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Induction of the photosystem 2 dark formation in etiolated leaves with the involvement of exogenous chlorophyllides

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Abstract

The delayed luminescence (DL) of photosystem 2 (PS2) after infiltration of 7-d-old etiolated barley leaves with chlorophyllides (Chlide) *a* or *b* followed by 2.5 h dark incubation was studied. Chlide *a* caused a very weak DL of PS2 just at the beginning of irradiation and the intensity of this DL was not higher when the infiltration medium contained 2 mM of NADPH. Chlide *b* was a somewhat more efficient inducer of PS2 formation in the dark and NADPH enhanced this efficiency 4.5 times though it did not affect the amount of esterified Chlides. The photoconversion of endogenous Pchlide led to a much higher intensity of the DL in comparison with the infiltration of Chlides, while the total amount of chlorophyll (Chl) formed was almost unchanged. The use of Chlide *b* together with the acetone extract from green leaves, devoid of pigments, resulted in the DL intensity comparable with that observed after Pchlide photoconversion followed by 2.5 h incubation in the dark. Dark formation of active PS2 in etiolated leaves was shown for the first time. Thus the dark formation of active PS2 may require Chl *b*, NADPH, and some unidentified water-soluble factor(s), synthesized in the dark after a short irradiation of etiolated leaves and inherent in green leaves.

Additional key words: chlorophyll; delayed luminescence; *Hordeum vulgare*; infiltration; NADPH; reaction centre.

Introduction

Dark-grown seedlings of angiosperms are unable to synthesize chlorophyll (Chl) and perform photosynthetic reactions. Their etioplasts accumulate the Chl precursor protochlorophyllide (Pchlide) and some protein components of the photosynthetic apparatus, *e.g.*, plastocyanin, cytochromes b_{559}, *f*, and b_6, ferredoxin, ferredoxin-

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Abbreviations: Chl - chlorophyll; Chlide - chlorophyllide; DL - delayed luminescence; Pchlide - protochlorophyllide; PS - photosystem; RC - reaction centre.

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$NADP^+$-reductase, the coupling factor of photophosphorylation, ribulose-1,5-bis-phosphate carboxylase/oxygenase, and the 33 and 24 kDa proteins of the oxygen-evolving complex (Kirk and Tillney-Bassett 1978, Radunz *et al.* 1986, Takabe 1986, Hashimoto *et al.* 1993).

Etioplasts may not accumulate nuclear-encoded Chl *a/b*-binding proteins and plastid-encoded Chl *a*-binding proteins (for a review see Hachtel and Friemann 1993). Nevertheless, the etioplasts contain the transcripts of plastid-encoded apoproteins of Chl-proteins (Hachtel and Friemann 1993), the P700-Chl-protein, and apoproteins of light-harvesting complexes (Nechushtai and Nelson 1985, Shlyk *et al.* 1986, White and Green 1987).

Irradiation influences the expression of a number of plastid and nuclear genes. Irradiation of dark-grown seedlings causes the accumulation of Chl *a* and Chl *a*-apoproteins (Apel and Kloppstech 1980, Vierling and Alberte 1983, Klein and Mullet 1986) and the development of activity of the photosynthetic apparatus, early manifestations of which were reviewed by Franck (1993). The accumulation of Chl *a*-apoproteins depends on Chl synthesis, which affects the stability of apoproteins (Hachtel and Friemann 1993, Rüdiger 1993). The direct role of Chl in stabilization of Chl apoproteins was demonstrated in pigmentless mutants (Herrin *et al.* 1992). Kim *et al.* (1994) and Eichaker *et al.* (1996) showed that supplies of exogenous Chlide to barley plastids and its esterification to Chl induced the accumulation of Chl proteins P700, CP47, CP43, D1, and D2 in plastids by enhancing the stability of Chl apoproteins.

All these results create a strong evidence of the key role of Chl in the assembly of PSs in greening plants. It remains unclear whether exogenous Chlide stimulates the assembly of functional PSs because the above-mentioned experiments did not test PS activities. An electron transfer through PS1 or PS2 can be used as a criterion of functional assembly of PSs. The activity of PS2 can be detected *in vivo* by delayed light emission (Strehler and Arnold 1951) referred to in this text as the delayed luminescence. We have previously shown that the DL of etiolated barley leaves exposed to radiation can be detected 6-10 min after conversion of Pchlide, and this is a manifestation of activity of the PS2 (Fradkin *et al.* 1982). In the present study, this method was used to investigate whether Chl synthesis from an exogenous precursor could induce the formation of active PS2 *in vivo* without any participation of radiant energy.

Materials and methods

Plants: Barley seedlings (*Hordeum vulgare* L. cv. Nadya) were grown in darkness at 22 °C for 7 d. The top 1 cm sections of their leaves were discarded and the adjacent 2.5 cm parts were used for the experiments.

Purification of chlorophylls: 7-d-old green leaves of barley were homogenized in 80 % acetone. The homogenate was filtered through two layers of cotton cloth, then Chls and carotenoids were extracted into hexane. Further the hexane was evaporated in a stream of warm air, and the pigments were redissolved in hexane with 1 % of

isopropanol and chromatographed on the column of 60 % of polyamide (*Ferak*, for TLC) and 40 % of cellulose (*Whatman*), using hexane-isopropanol mixtures (from 2 to 20 %) as a developing solvent. Under these conditions, Chls *a* and *b* were separated and purified from carotenes, xanthophylls, and pheophytins. If either Chl species was free from impurities of the other one was checked by confirming the absence of shoulders in the absorption spectrum at 460 nm (for Chl *a*) and 665 nm (for Chl *b*). The bands of carotenoids in the blue region of spectrum were absent. The purified pigments were dried, dissolved in acetone, and used for preparation of Chlides.

Preparation of chlorophyllides: Chlides *a* and *b* were prepared by hydrolysis of purified Chls *a* and *b* in acetone (60 %) with 50 mM Tris-HCl buffer pH 8 (40 %) at 37 °C for 2.5 h, using rye chlorophyllase isolated from etiolated leaves. After the incubation, the slurry was filtered through two layers of cotton cloth, and acetone was added to the filtrate to make a final concentration of 80 %. The acetone extract was washed with hexane to remove Chls. Then, Chlide was extracted with diethyl ether, dried, redissolved with a fresh portion of ether, and kept at -12 °C until the start of an experiment. For the experiment, a portion of the solution of Chlide was dried and dissolved in distilled water containing NH_4OH at pH 7.2. This aqueous solution was used for infiltration into etiolated barley seedlings. The concentration of Chlide in the solution was determined spectrophotometrically.

Pigmentless fraction of acetone extract: Green barley leaves were homogenized and subsequently extracted with 80 % acetone. The extract was washed with hexane and then with diethyl ether to remove all pigments. The colourless product was dried, redissolved in distilled water, neutralized with NH_4OH to pH 7.2, designated as a "pigmentless fraction", and used as an addition to infiltration medium. The ratio between the added portion of pigmentless fraction and the portion of Chlide was consistent with their contributions into the original extract before removing the pigments.

Vacuum infiltration: For infiltration, identical pieces of leaves were placed into a syringe together with the infiltration solution. After several low-high pressure cycles, induced by movements of the piston, the intercellular spaces were filled with the solution. The leaf surfaces were dried with filter paper and the leaves were kept in the dark for 2.5 h. All procedures were carried out in a dim green radiation.

PS2-activity measurement: The DL of leaves was determined with a cylindrical phosphoroscope (Domanskiĭ *et al.* 1986). In the dark, ten seedlings were attached to a plastic plate and placed into the device. Then an exciting radiation was switched on for 10 s and the DL intensity was recorded. The intensity of the red exciting radiation (cut-off wavelength 650 nm) was 15 W m^{-2}. The duration of "excitation-registration" cycle was 10 ms.

Pigment assay: Following the dark incubation, five leaves of each sample were homogenized in 80 % acetone. Chls were extracted twice with hexane, then Chlides were transferred from the acetone solution into diethyl ether. Pigment concentrations were determined spectrophotometrically with a *Uvicon-931* spectrophotometer

(*Contron Instruments*, Germany). The pigment contents were calculated using the equations of Brouers and Michel-Wolwertz (1983).

Results

The delayed luminescence of etiolated leaves infiltrated with Chlides: Under the experimental conditions, Chlide *b* but not Chlide *a* caused an appearance of PS2 activity, detected by its DL after 2.5 h dark incubation (Fig. 1). This effect was more noticeable in the presence of 2 mM NADPH.

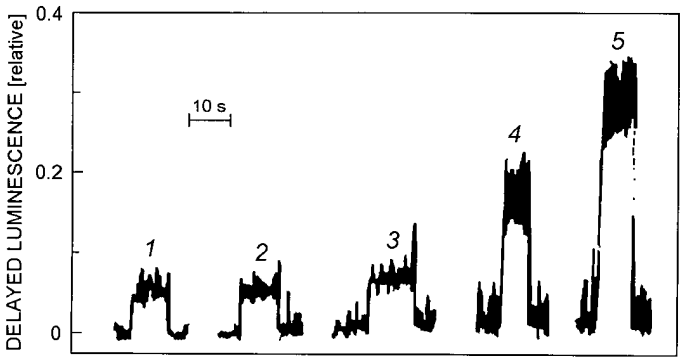

Fig. 1. Intensity of delayed luminescence of photosystem 2 after 2.5 h dark incubation of etiolated barley leaves previously infiltrated with water solution (alkalified to pH 7.2) of: *2* - Chlide *a*, *3* - Chlide *a*+NADPH, *4* - Chlide *b*, *5* - Chlide *b*+NADPH. *1* - Emission of noninfiltrated leaves characterizes the zero level of photosynthetic luminescence. Concentrations of Chlides were 0.07 kg m^{-3}, and of NADPH 2 mM.

To clarify whether it is possible to induce the appearance of the PS2 activity with Chlide *a* and what in this respect are the relative capacities of both Chlides, the leaves were infiltrated with one or another Chlide over a wide range of their concentrations. Both Chlides caused formation of active PS2, but Chlide *b* was much more efficient than Chlide *a* (Fig. 2). Hence during the experiment shown in Fig. 1 Chlide *a* failed to trigger the appearance of DL emitting centres because of the low pigment amount used. The dependence of DL intensity on Chlide concentration was nonlinear. The scatter in values of three performed experiments did not permit to trace the exact course of curve 2 and thus its shape for low Chlide concentrations was drawn arbitrarily. Nevertheless, obviously at Chlide concentrations higher than 0.07 kg m^{-3} the growth of the Chlide concentration increased the DL intensity more than that at lower concentrations.

The yields of Chlide esterification and intensities of delayed luminescence: The difference in the actions of Chlides may be due to their different abilities to penetrate to their natural location in plastids and/or to different efficiencies in esterification to

Chl. To examine this point, the pigment composition of infiltrated leaves was studied using the absorption spectra of esterified and nonesterified fractions.

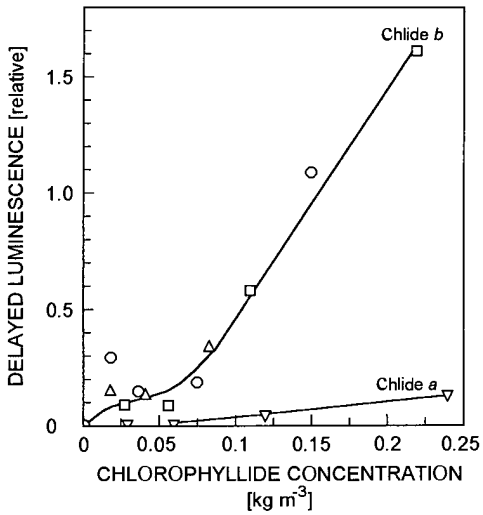

Fig. 2. Intensity of delayed luminescence of photosystem 2 of etiolated leaves infiltrated with alkalified water (pH 7.2) with different concentrations of Chlide *a* or *b* and 2 mM NADPH. Three experiments presented by different symbols.

Comparison of Chl amounts with the total amounts of recovered Chls and Chlides and with the DL intensities for different treatments (Table 1) showed that the yields of Chls were fairly similar for the leaves infiltrated with Chl *a* or Chl *b* but the relative amounts of the two Chls were very different. The Chl *a*/*b* ratio in the leaves infiltrated with Chlide *a* was very high. In Table 1 only the lower limit of this ratio is given, since so small relative amounts of Chl *b* cannot be calculated properly from the absorption spectra.

Table 1. Pigment composition and photosystem 2 delayed luminescence of 7-d-old etiolated barley leaves after infiltration with different agents and following incubation for 2.5 h in the dark. The concentrations of both chlorophyllides (Chlides) were 0.23 kg m^{-3}, 2 mM NADPH. In the last variant leaves were infiltrated with water only, then irradiated by "white light" of 20 W m^{-2} for 30 s and incubated in the dark. Chl - chlorophyll.

Agent	Recovered Chlide [nmol $leaf^{-1}$]	Chl (*a*+*b*) formed [nmol $leaf^{-1}$]	Chl *a* formed	Chl yield [%]	Chl *a*/*b*	DL intensity	DL per Chl	DL per Chl *a*
Chlide *a*+NADPH	2.39	0.33	0.33	13.8	>50	0.12	0.36	0.36
Chlide *b*	1.73	0.18	0.12	10.4	2.0	0.08	0.44	0.67
Chlide *b*+NADPH	2.00	0.24	0.15	11.7	1.7	0.45	1.90	3.00
Water, irradiated	0.50	0.25	0.22	71.5	7.4	5.90	23.6	26.80

Table 1 enables four statements: (*1*) In all experimental variants an appreciable part of inserted Chlide was esterified to Chl, mainly to Chl *a*, even if the leaves were infiltrated only with Chlide *b*. This was due to the "reverse reaction" of Chl formation (Rudoĭ *et al.* 1977). (*2*) At similar amounts of formed Chl, the DL intensities were very different. In the presence of NADPH the introduction of Chlide *b* caused a more intense DL even though the amount of Chls, especially of Chl *a* (the pigment of reaction centres, RCs), was higher after introduction of Chlide *a*. Thus Chlide *b* was a more efficient inducer of PS2 activity than Chlide *a*. (*3*) The presence of NADPH had a little effect, if any, on Chl formation, but it increased the activity of PS2 more markedly. (*4*) After the natural photoinduced formation of Chlide, the DL of PS2 is almost an order of magnitude higher in comparison with the infiltration experiments despite very similar amounts of produced Chl.

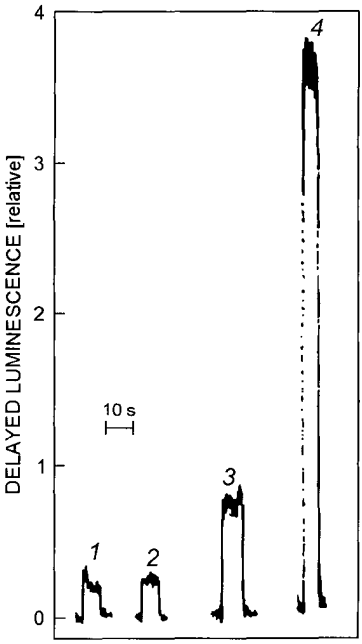

Fig. 3. Intensity of delayed luminescence of photosystem 2 after dark incubation of etiolated barley leaves, infiltrated with alkalified water (pH 7.2) containing: *1* - Chlide *a*, *2* - Chlide *a*+pigmentless fraction from the acetone extract from green barley leaves, *3* - Chlide *b*, *4* - Chlide *b*+pigmentless fraction. Concentration of both Chlides was about 0.2 kg m^{-3}.

Some factor from irradiated plants enhances the intensity of DL of PS2 after its introduction into etiolated leaves simultaneously with Chlide: Fig. 3 shows the records of DL for etiolated leaves infiltrated with pure Chlide *a* or *b* together with the pigmentless fraction. Here the concentration of Chlide was higher than during the experiment shown in Fig. 1 and also the DL signals were more pronounced. The DL intensity of the leaves infiltrated with Chlide *a* was very low and almost independent on the additive. On the contrary, Chlide *b* induced a noticeable activity of PS2,

especially in the presence of the "pigmentless fraction". In the latter case the intensity of DL was only half as high as that after photoconversion of endogenous Pchlide. We did not detect any significant influence of the pigmentless fraction on the amount of synthesized Chl (values not shown).

In an attempt to identify the factor that was present in the pigmentless fraction and promoted dark biogenesis of PS2, further experiments were made. They showed that the active factor (or factors) is water-soluble and insoluble in dry acetone. It can be concentrated by DEAE-cellulose chromatography, so presumably it is an anion. Because of its temperature stability (it was not destroyed even at 80 °C) it is unlikely to be a protein.

Discussion

Formation of Chls in etiolated leaves infiltrated with Chlides: Generally, incorporation of Chlide into etiolated leaves (Rudoï and Vezitskiï 1976, Rudoï *et al.* 1977) or into etioplasts (Eichacker *et al.* 1990) leads to the synthesis of Chl in darkness. Herein, exogenous Chlides were esterified in the leaves with a rather high efficiency. The amounts of Chls were comparable to those found in the leaves after photoreduction of Pchlide (Table 1).

Judging from the absorption spectra, mutual transformations of Chlides did not occur in infiltrated leaves with appreciable efficiency which agrees with the results of Rudoï *et al.* (1977). The conversion of Chl *b* to Chl *a* was highly efficient and Chl *a* to Chl *b* transformation was very weak, so that a negligible, if any, amount of Chl *b* was found after the introduction of Chlide *a* (Table 1).

Etiolated leaves are able to form active PS2 in the dark when supplied with Chlide plus cofactors: Irradiation of etiolated leaves results in an appearance of quickly rising DL emitted by developing PS2 (Fradkin *et al.* 1982, Shlyk *et al.* 1985). The ability of etiolated leaves infiltrated with Chlide plus cofactors to emit the DL immediately at the beginning of irradiation indicates the formation of active PS2 in the dark. It is the first evidence of formation of active RCs of PS2 in darkness in the presence of Chl.

The present results show that Chlide *b* supplied to a leaf together with NADPH or the pigmentless fraction is an efficient inducer of PS2 formation. The formation of phytol and, consequently, the Chl esterification needs NADPH (Rüdiger 1993). The first stage of the Chl *b* to Chl *a* conversion, formation of 7-hydroxymethylchlorophyll also requires NADPH (Itoh *et al.* 1996). In our experiments the yield of Chls, of Chl *a* in particular, after addition of Chlide *b* was slightly affected by NADPH. This indicates that NADPH influences the processes of biosynthesis and assembly of PS2 components, which were induced by the Chl formation.

The NADPH concentration in the infiltration medium was adjusted so that it was close to the maximum amount of NADPH in the pigmentless fraction estimated by the absorption spectrum. Thus a weak action of NADPH on DL of PS2 as compared to the action of the pigmentless fraction can not be explained by a low NADPH concentration. Hence, the pigmentless fraction extracted from green plants must

contain a factor that is absent from etiolated leaves and is also able to stimulate the formation of PS2.

Chl from exogenous Chlide was less efficient in inducing the ability to emit DL than Chl that was synthesised from endogenous Chlide (Table 1). Such a result may mean that under irradiation some factor(s) arise that facilitate the assembly of active PS2. This could explain some our previous findings (Shlyk *et al.* 1985) in which the DL of PS2 was larger at a higher irradiance even during the lag-phase of Chl accumulation in greening barley leaves, when the amount of Chl did not depend on the supply of radiant energy.

Evidently, a share of Chl molecules introduced into the leaf as well as other participants of electron transfer were bound by apoproteins of RCs of PS2, and active RCs of PS2 were assembled in the dark. Because CP47 and CP43 accumulate in barley etioplasts supplied with Chlide (Eichacker *et al.* 1996), the core of PS2 may also be assembled in etiolated leaves infiltrated with Chlide.

A special role of Chl *b* in induction of assembly of PS2 RCs: The RCs of both PSs contain Chl *a*, but not Chl *b*. All values obtained in the present work indicate that Chlide *b* is a much more efficient inducer of formation of PS2 than Chlide *a*. This clear dependence of the PS2 formation on the presence of Chl *b* suggests a special role of Chl *b* in the induction of the PS2 assembly. It can be proposed, for example, that some Chl *a/b*-binding protein (similar to LHC-apoprotein) needs both Chls for its stabilization and/or translocation of Chl *a* to sites of formation of PS2. Bellemare *et al.* (1982) have already suggested that Chl *b* was required to stabilize the Chl *a/b*-binding polypeptides.

The fact that Chl *b*-less mutants form functionally active PS2 and are viable suggests that the presence of Chl *b* is an important but not obligatory factor for the efficient formation of PS2.

During normal greening of etiolated leaves, a fast build-up of Chl *b* starts after a continuous irradiation of leaves in parallel with accumulation of Chl *a/b*-binding protein. However, Fradkin *et al.* (1966) have shown that Chl *b* appears also in the dark after a short irradiation of etiolated leaves. The immediate appearance of Chl *b* at the very beginning of irradiation and in the dark after a short radiation pulse was described by Shlyk *et al.* (1970). During the first hour of greening of etiolated barley leaf most Chls resided out of the two PSs but Chl molecules were gradually rearranged from this intermediate pool into the two photosystems (Fradkin *et al.* 1982, Shlyk *et al.* 1985). Now it is generally recognized that Chl molecules are always bound to some protein. As Chl *b* already exists in this period, both Chls are presumably associated with some Chl *a/b*-binding protein from the outset of greening. The function of such transient carrier of pigments has been postulated for early light-induced proteins (Adamska 1995) and for the PS2-S protein (Funk *et al.* 1995). Moreover, Adamska (1995) suggested that ELIP (the protein of light stress) in pea was a transient component of PS2. Kuttkat *et al.* (1997) assumed that Chl carrier proteins could shuttle "Chls to the site where they are assembled in newly formed pigment-protein complexes". It is likely that ELIP or another protein with Chl *b*-

binding ability is a transient carrier for Chl molecules on their way to the photosynthetic apparatus after the darkness was changed by light.

It cannot be excluded that the temporary stay of Chl on such a transient carrier is the reason of the essential delay in appearance of DL of PS2 at the beginning of greening (Shlyk *et al.* 1985, Domanskiĭ and Fradkin 1992).

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