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Communication

Phytochrome Effects on the Relationship between Chlorophyll and Steady-State Levels of Thylakoid Polypeptides in Light-Grown Tobacco[1]

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ABSTRACT

The effects of phytochrome status on chlorophyll content and on steady-state levels of thylakoid proteins were investigated in green leaves of *Nicotiana tabacum* L. plants grown under white light. Far-red light given either as a pulse at the end of each photoperiod, or as a supplement to white light during the photoperiod, reduced chlorophyll content per unit area and per unit dry weight. These differences were also observed after resolving chlorophyll-containing polypeptides by gel electrophoresis. Chlorophyll *a*:*b* ratio was unchanged. Both Coomassie blue-stained gels and immunochemical analyses showed that, in contrast to the observations in etiolated barley (K Apel, K Kloppstech [1980] Planta 150: 426–430) and pea (J Bennett [1981] Eur J Biochem 118: 61–70) seedlings, and in etiolated tobacco leaves (this report), in fully deetiolated tobacco plants changes in chlorophyll content were not correlated with obvious changes in the steady-state levels of thylakoid proteins (*e.g.* light-harvesting, chlorophyll *a*/*b*-binding proteins).

In etiolated seedlings of *Hordeum vulgare*, a R[3] pulse triggers the appearance of mRNA activity for the LHCP (2). The message is taken up into the polysomes in subsequent D and may be translated *in vitro* in a cell-free protein synthesizing system (2). However, an accumulation of freshly synthesized polypeptide within the plant is not observed (2). Similarly, when etiolated pea seedlings are exposed to continuous light for 24 h and then returned to D, 38% of the Chl *a*, 74% of the Chl *b*, and 84% of the LHCP that had accumulated under illumination are unstable in D (3). These losses occur despite the fact that LHCP synthesis continues for up to 48 h after transfer to D (3).

The apparent instability of LHCP might be explained by a deficiency of Chl in R-treated barley plants, or by the degradation of preexisting Chl in pea plants returned to D (2, 3, 9). Particularly, Chl *a* seems to be the light-dependent factor which is required for the stabilization of the LHCP (2). Protein turnover could be part of the normal physiological mechanism for coordinating the accumulation of the pigment and protein components of the light-harvesting Chl *a*/*b* complex (3).

Most of our knowledge on phytochrome-mediated effects at the molecular level comes from experiments using etiolated seedlings. The present experiments were carried out as part of a project aimed at investigating this issue in WL-grown plants, where a different form of phytochrome predominates (1, 20). Taking into account the effects of Pfr/P on Chl levels in WL-grown plants of different species (5, 12, 15, 19), and previous results with etiolated seedlings (2, 3, 9), we expected to observe differences at the level of thylakoid proteins in WL-grown plants of these species, and use the system for further studies. However, rather unexpected results were found and are reported in this paper.

MATERIALS AND METHODS

Plant Material and Light Conditions

Plants of *Nicotiana tabacum* L. cv SR1 were grown in a glasshouse for 1 month. For the experiments in which Pfr/P was modified only at the end of each photoperiod, plants were transferred to a growth room providing a PPFD of 220 μmol m^{-2} s^{-1} (6). Photoperiod was 8 h and temperature 22°C. Before D, the plants received 10 min R (100 μmol m^{-2} s^{-1}, Pfr/P = 0.84, from low pressure sodium lamps [7]) or FR pulses (102 μmol m^{-2} s^{-1}, Pfr/P = 0.01, from incandescent lamps in combination with water and black acrylic filters [6]), or remained as controls, without further irradiation after WL. In other experiments, the Pfr/P provided by WL was modified by supplementary FR in a growth chamber (Pfr/P = 0.7 and 0.5 for WL and WL + FR, respectively [6]). The PPFD was 75 μmol m^{-2} s^{-1}, photoperiod 16 h and temperature 24°C. Sampled leaves had already expanded and greened at the beginning of treatments, unless stated otherwise.

Chl Determinations, Green and Coomassie Blue-Stained Gels

Chl *a* and *b* were measured in *N*,*N*-dimethylformamide extracts from three 0.16 cm^2 leaf discs per plant (5, 11). Dry

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[3] Abbreviations: R, red light; LHCP, light-harvesting, Chl *a*/*b*-binding protein(s); D, darkness; WL, white light; Pfr/P, calculated phytochrome photoequilibrium; FR, far-red light; TBS, 50 mM Tris-HCl (pH 7.4), 200 mM NaCl.

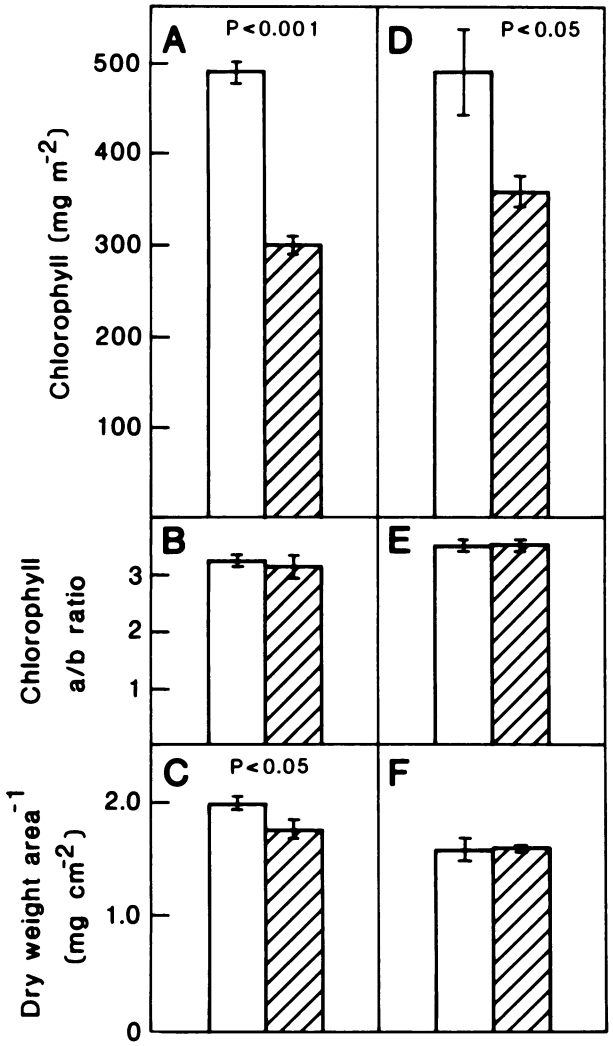

Figure 1. Effects of an FR pulse at the end of each photoperiod (A, B, C), and effects of supplementary FR during the photoperiod (D, E, F), on Chl content per unit area (A, D), Chl *a*:*b* ratio (B, E), and dry weight per unit area (C, F). Dashed bars: end-of-day or daytime FR; open bars: WL controls. Treatments started when sampled leaves were fully expanded. Plants were harvested after 12 d treatments. Data are means of seven (A, B, C) or four (D, E, F) plant replicates ± SE.

weight per unit area was measured for three 3.46 cm^2 leaf discs per plant. Thylakoids were isolated according to Roberts *et al.* (17). Twenty-five 3.46 cm^2 discs per plant were homogenized, in ice-cold 0.3 M sorbitol, 50 mM $MgCl_2$, 1% (w/v) BSA, 50 mM Tricine (pH 8.0), by one 10-s burst using a kitchen blender. The homogenate was filtered through eight layers of muslin and chloroplasts pelleted by centrifugation for 2 min at 2000*g*. The pelleted chloroplasts were washed twice by resuspension in 0.3 M sorbitol, 50 mM Tricine (pH 8), and centrifugation for 2 min at 2000*g*. Washed chloroplasts were lysed by resuspension in 50 mM Tricine (pH 8), and incubation on ice for 10 min. Plastid membranes were pelleted by centrifugation for 5 min at 6000*g*, and resuspended in 1 mL wash buffer. For protein measurements, 300 μL of thylakoid suspension were precipitated in 30% TCA and washed according to Roberts *et al.* (17). Protein was determined by the method of Ghosh *et al.* (10). For PAGE, the remaining thylakoid suspension (700 μL) was pelleted by centrifugation at high speed in a microcentrifuge for 10 min. The pellet was resuspended in distilled water using a glass rod, and further diluted up to 270 μL. Thirty μL of 10% SDS in 1.5 M Tris/H_2SO_4, pH 9 buffer, were added (14) and, after 20 min at room temperature, the mixture was centrifuged at high speed in a microcentrifuge for 20 min. This resulted in a small pellet of nonsolubilized material and a dark green translucent supernatant that was used immediately for PAGE. SDS-PAGE was carried out according to Laemmli (13) in 12.5%, 1.5 mm thick, gels using Mini-Protean II Dual slab cells (Bio-Rad). The gels were run for 45 min at 200 V. Low mol wt standards from Bio-Rad were used. Gels were stained with 0.025% (w/v) Coomassie blue R in 50% (v/v) ethanol, 5% (v/v) acetic acid, (60 min at 37°C), and destained with 7.5% (v/v) acetic acid, 5% (v/v) methanol. Both green- and Coomassie blue-stained gels were scanned in a GS 300 Hoefer densitometer (Hoefer Scientific Instruments, San Francisco, CA). Protein bands were cut from fixed gels after washing in distilled water for 24 h, loaded in a gel (13) with a 5 cm stacking gel, and reelectrophoresed overnight (70 V) to aid protein identification after staining and destaining as indicated above.

Preparation of Antiserum

A thylakoidal pellet was obtained from cotyledons from 4-d-old, light-grown mustard (*Sinapis alba* L.) seedlings, as

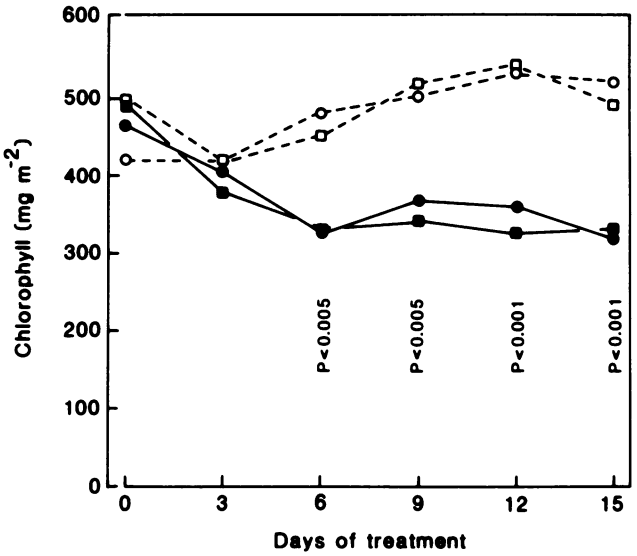

Figure 2. Effects of R (○), FR (●), R followed by FR (■), and FR followed by R (□) pulses given at the end of the photoperiod, on Chl content per unit area. Data are means of three plant replicates. Treaments started when sampled leaves were fully expanded. Data were analyzed using factorial analysis of variance, factors were final Pfr/P (R *versus* FR as the last pulse), and presence *versus* absence of a previous pulse. The significance for the final Pfr/P is indicated, the other main effect and the interaction were not significant ($P > 0.20$).

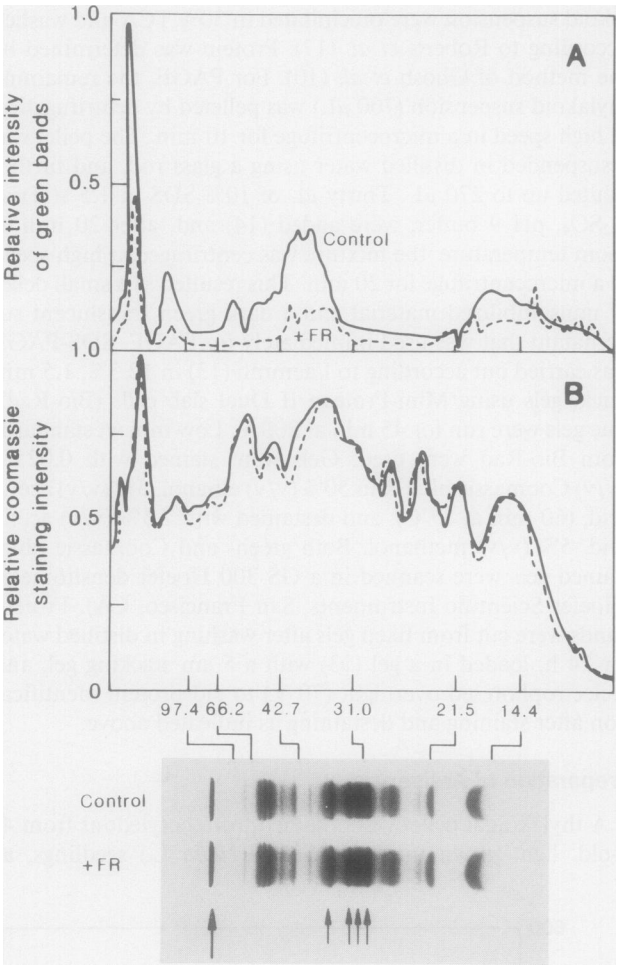

Figure 3. Effects of a FR pulse given at the end of each photoperiod on the intensity of Chl containing bands (A), and thylakoid proteins stained with Coomassie blue (B), after PAGE. Treatments started when sampled leaves were fully expanded. The abscissa shows the migration of molecular mass markers (kD). A typical Coomassie blue-stained gel is shown at the bottom: long arrow = P700, short arrows = LHCP. Equal volumes (8 μL) of the final supernatant were loaded (total protein concentration in the supernatant was unaffected by Pfr/P).

described for tobacco. The pellet was washed twice by resuspension in 0.3 M sorbitol, 5 mM $MgCl_2$, 10 mM Tricine (pH 7), and centrifugation at 6000*g* for 5 min. Thylakoid proteins were solubilized in SDS sample buffer and electrophoresed on a preparative 8 to 16% (w/v) gradient polyacrylamide gel. Following brief staining with Coomassie blue, the major band at about 26 kD was excised from the gel, rinsed in distilled water, equilibrated in SDS sample buffer, and subjected to a second electrophoresis. The polypeptide band in this gel, visualized by soaking the gel in ice-cold 0.1 M KCl, was excised and the protein eluted by homogenizing the gel slices in 50 mM sodium phosphate buffer (pH 7.4), 0.15 M NaCl, 0.1% (w/v) SDS, and incubating the crushed gel for 48 h at 4°C. The gel fragments were removed by centrifugation, the supernatant lyophilized and dissolved in 50 mM sodium phosphate buffer, 0.15 M NaCl, 0.1% (w/v) SDS, to a concentration of 1 mg mL^{-1}.

New Zealand white rabbits were immunized with 0.5 mL of resuspended protein mixed with an equal volume of Freund's complete adjuvant by multiple subcutaneous injections. After 2 and 4 weeks the rabbits were given booster injections of 250 μg protein mixed with Freund's incomplete adjuvant. The rabbits were bled, by cardiac puncture, 14 d after the final injection. A crude immunoglobulin fraction of the serum was prepared by repeated precipitation with 50% saturated ammonium sulphate, followed by resuspension of precipitated protein in 50 mM sodium phosphate buffer (pH 7.4), 0.15 M NaCl and dialysis against the same buffer. The antisera were tested by Western blotting and found to immunostain only the 26 kD thylakoid polypeptide. This polypeptide was not detectable on Western blots of soluble chloroplast proteins, nor on Western blots of etioplast membrane proteins (data not shown).

Western Blots

Leaf samples were lyophilized and reduced to a fine powder. Twenty mg of powder were solubilized in 500 μL SDS sample buffer, incubated at room temperature for 5 min, and boiled for 2 min. After centrifugation at 13,000*g* for 10 min, aliquots of the supernatant were used for SDS-PAGE and total protein determinations as described above. After SDS-PAGE, proteins were electroblotted (Milliblot-SDE System, Millipore) on nitrocellulose filters. Nitrocellulose sheets were incubated for 30 min at 37°C in 5% dry milk powder (Marvel) dissolved in TBS and 0.1% Tween 20. Blots were then incubated

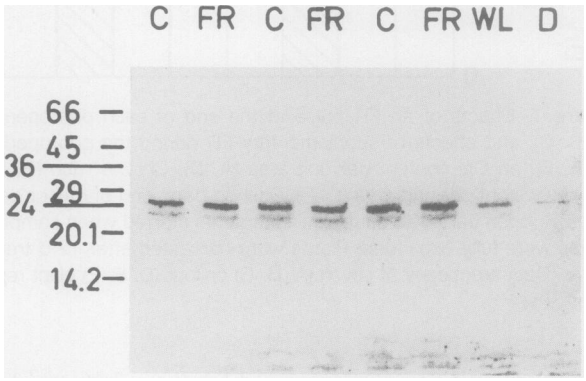

Figure 4. Immunoblot analysis of extracts from tobacco leaves from control (C) or end-of-day FR-treated plants (FR). Different Pfr/P treatments started when sampled leaves were fully expanded. Electroblotted proteins were probed with rabbit anti-*S. alba* LHCP. Three independent extracts are shown for each light condition. The two right hand lanes show extracts from expanding leaves of plants either cultivated under WL photoperiods (WL) or transferred to continuous D for 10 d before the harvest (D) in order to compare the effects of different degrees of deetiolation. The numbers show the migration of molecular mass markers (kD). Equal volumes (5 μL) of 20 mg of leaf powder dissolved in 500 μL SDS sample buffer were loaded (total protein concentration in the supernatant was unaffected by the light treatments). The magnitude of Pfr/P effects of Chl content was as shown in Figure 1.

overnight (4°C) in anti-LHCP antibody (1:500 dilution) in 1% dry milk powder in TBS, rinsed three times with TBS-Tween 20, then incubated for 1 h in 1:500 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody in TBS and 1% dry milk, and again rinsed several times with TBS-Tween 20. The blots were then allowed to react with the developer (0.1 M Tris [pH 9.5], 1 mM $MgCl_2$ containing 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indoyl phosphate, *p*-toluidine salt), and finally washed with distilled water. Two bands corresponding to LHCP were observed in both tobacco and mustard samples.

Statistics

Experiments were repeated and the final number of replicates (independent extractions) pooled, and analyzed by analysis of variance, after using Bartlett's test for homogeneity of variance. Plant position in the growth room was random and the growth-chamber compartment with FR-enriched WL was alternated in different experiments. Green- and Coomassie blue-stained gels are representative from six replicates. Ten Western blots were carried out under a range of conditions using four independent extractions per treatment. For Coomassie blue-stained gels and Western blots, different protein concentrations were used to ensure that the level of staining was in the range sensitive to changes in protein concentration.

RESULTS

Both a FR pulse at the end of each photoperiod and supplementary FR during the photoperiod, reduced Chl concentration of fully expanded leaves (Fig. 1, A, D), while Chl *a*:*b* ratio was unchanged (Fig. 1, B, E) and dry weight per unit area was either slightly affected or unchanged (Fig. 1, C, F). The effects on Chl content were mediated by phytochrome perception of the light signals and were significant within 6 d from the beginning of treatments (Fig. 2). Different Pfr/P also affected Chl content of unfolding greening leaves without changing the Chl *a*:*b* ratio (*e.g.* for experiments involving end-of-day pulses, Chl [mg m^{-2}] = R: 331; FR: 229; $P < 0.001$; Chl *a*:*b* ratio = R: 3.8; FR: 4.0; $P > 0.2$; $n = 9$).

Three major Chl-containing regions were separated by PAGE (Fig. 3A) (16). The first one, with an apparent molecular mass higher than 100 kD, contains the P700-Chl *a* complex (16). The antenna of this band does not fluoresce under UV (data not shown) (14). The second zone, with an apparent molecular mass between 30 and 50 kD, contains Chl *a* and *b* (16), and showed four discrete bands which were not fully resolved in the scans. The third band, with an apparent molecular mass lower than 21.5 kD contains free Chl complexed with SDS, and carotenoids (16). Several polypeptides were revealed after staining with Coomassie blue, including P-700 and LHCP (Fig. 3B).

Lowering Pfr/P at the end of each photoperiod reduced Chl content of all the bands (average reduction was 40%, Fig. 3A). These differences were not correlated with obvious differences in protein steady-state levels as indicated both by Coomassie blue-stained gels (Fig. 3B), and immunochemical analysis for LHCP (Fig. 4). On the contrary, young leaves of plants transferred to continuous D, showed reduced levels of both Chl (WL: 168 mg m^{-2}; D: 33 mg m^{-2}) and LHCP (Fig. 4) compared to leaves expanding under normal photoperiods. The latter agrees with previous observation in etiolated seedlings of other species (2, 3, 9).

DISCUSSION

These results indicate the following: (a), in light-grown tobacco (as previously shown for *Petunia axillaris*, [4, 5]) low Pfr/P reduces Chl content of fully greened leaves (Fig. 2), without changing the Chl *a*:*b* ratio (Fig. 1, b, e); (b) changes in Chl *a* content of similar magnitude to those reported to cause the destabilization of LHCP in deetiolating pea (3) are not correlated with obvious reductions in LHCP steady-state levels in WL-grown tobacco.

Phytochrome-deficient mutants of *Arabidopsis thaliana* seedlings grown under WL have reduced levels of both Chl and LHCP (8). In principle, this difference with our results could be explained by considering that in the mutants, low Pfr levels were present during the whole greening period, but the light treatments used here established differences in Pfr/P and Chl content, only after the sampled leaves were fully greened.

Sagar *et al.* (18) reported that changes in Chl content caused by herbicide bleaching were not strictly correlated with changes in LHCP amounts. However, in contrast to herbicide treatments, phytochrome-mediated effects are part of normal plant physiology under natural conditions. Therefore, the coordination (at least the rapid coordination) between Chl and thylakoid protein components found in pea and barley seedlings during deetiolation (2, 3, 9) is not obvious in fully deetiolated tobacco.

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