Early Light-inducible Protein in Pea Is Stable during Light Stress but Is Degraded during Recovery at Low Light Intensity*

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The early light-inducible proteins (ELIPs) are among the first light induced nuclear-encoded proteins to be detected in the developing chloroplast membranes (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987). The ELIPs are synthesized on cytosolic ribosomes as precursor proteins (Grimm et al., 1989). Based on the homology of ELIP with the cab proteins it was proposed that ELIP may be involved in the early stages of chlorophyll a/b-protein assembly (Grimm and Kloppstech, 1987; Grimm et al., 1989). The induction of ELIP mRNA synthesis and accumulation of its translation product in developing plants was shown to be under diurnal and circadian control (Kloppstech, 1985; Otto et al., 1987). Recent results obtained in our laboratory have shown that in green plants ELIP transcription is induced specifically by blue (Adamska et al., 1992b) and UV but not UVB light (Adamska et al., 1992a).

Cross-linking experiments have demonstrated that ELIP transferred in vitro into intact chloroplasts under high light conditions interacts with the D1 protein of the photochemical reaction center of photosystem II (Adamska and Kloppstech, 1991).

So far no definite function was described for ELIP in higher plants. The proximity of ELIP to PSII and the known fact that this photosystem is sensitive to high light intensities have prompted us to test whether ELIP is induced as a light stress response. The results so far obtained indeed supported this concept (Adamska et al., 1992a, 1992b).

In the present work ELIP mRNA and protein turnover in plants exposed to white light stress and during the recovery process have been investigated. The results demonstrate that ELIP is synthesized in mature pea plants exposed to light stress. The mRNA is unstable, however, the protein does not rise above the level which induces its synthesis. This low light-induced protein degradation was so far not described for proteins related to the light stress process.

MATERIALS AND METHODS

Growth of Plants and Illumination Conditions—Pea plants (Pisum sativum L. cv. Alaska) were grown for 14 days on vermiculite at 25 °C at a light intensity of 40 μE/m²s provided by blue fluorescent lamps and under light regime of 12 h dark/12 h light (light from 0800-2000).

The high light treatment was performed on detached mature leaves floated on water. High light intensity was provided by tungsten-halogen lamps (2000 watts). The plants were protected from excess infrared radiation by a 3-cm double-walled glass screen cooled by a water-circulating system. The ambient temperature near the plants or that of the water on which the detached leaves were floated did not rise above 27 °C.

Treatment with Carotene Synthesis Inhibitors—To inhibit carotenoid biosynthesis in high light the following herbicides were used during illumination at concentrations as mentioned: 20 μM fluridone (1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4(1H)-pyrindo), 100 μM norflurazon (4-chloro-5-(methylamino)-2-[5-(trifluorome-
thyl)phenyl-3(2H)-pyridazinone), and 10 μM pyrimidine derivate KM 145-155.

Isolation of Poly(A')-RNA and in Vitro Translation—Poly(A')-RNA was prepared according to Logemann et al. (1987) using oligo(dT)-cellulose chromatography (Pemberton et al., 1975). In vitro translation was performed using the wheat germ system according to Roberts and Paterson (1973) in the presence of [35S]methionine (Amersham). For immunoprecipitation 40 μl of in vitro translation assay was diluted to a 200-μl final volume and incubated with anti-ELIP antibody at 1/40 dilution overnight in the cold. Antibody-protein conjugates were precipitated by addition of 30 μl of preswollen protein A-Sepharose for 2 h with constant shaking followed by centrifugation for 10 min at 12,000 x g. The resulting pellet was analyzed by SDS-PAGE followed by autoradiography.

Dot Blot Hybridization—Insert of an ELIP cDNA clone (Scharnowsky et al., 1985) was labeled using random oligonucleotides as primers according to Boehringer Mannheim protocol. Dot blot hybridization was performed as described before (Otto et al., 1988).

In Vivo Labeling and Isolation of Thylakoid Membranes—For in vivo labeling, detached leaves were floated on water with addition of 100 μCi/ml [35S]methionine (1220 Ci/mmol, Du Pont-New England Nuclear) during the light treatment. At the end of the incubation the leaves were frozen in liquid nitrogen and further used or stored at −80 °C. Membranes isolation the frozen plant material was ground in a liquid nitrogen-precooled mortar, and the tissue powder was resuspended in 2 ml of double-distilled water in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM aminocaproic acid). The suspension was filtered through two layers of nylon nets (125 and 40 μm) to remove broken cells and debris, and 4 ml NaCl was added to the final concentration of 0.24 M to induce complete membrane stacking and facilitate sedimentation of all thylakoid membranes at 12,000 x g for 10 min. The pellet was washed once in the presence of protease inhibitors and then resuspended at a final concentration of 10–15 mg protein/ml in sample buffer prior to electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed according to Laemmli (1970) using the Hoeffer minigel system. Radioactive gels were dried and exposed to x-ray film at −70 °C. Immunoblotting (Towbin et al., 1979) was carried out using 40-μm pore size nitrocellulose paper. Blots were incubated with antibodies and detected by the enhanced chemiluminescence method (Amersham).

Pigment Analysis—Chlorophyll content was determined spectrophotometrically in 80% acetone extracts according to the method of Arnon (1949).

Pigments (carotenoids and chlorophylls) were extracted after homogenization of leaves with acetone followed by filtration. This process was repeated several times until complete removal of all pigments. The pigment solution was mixed with petrothel in a separation funnel and following phase separation the pigment-containing phase was washed 1× with 4 M NaCl and 2× with H2O. The petrothel was dried with Na2SO4 and evaporated under vacuum, and the residual pigments were dissolved in 1 ml of acetone.

HPLC analysis was performed as described by Humbeck et al. (1989).

Measurements of Variable Fluorescence (Fv)—Thylakoid membranes were suspended (17 μg of chlorophyll/ml) in 50 mM Tricine buffer (pH 7.8) containing 10 mM NaCl and 5 mM MgCl2. The suspension was assayed for the fluorescence rise kinetics using the apparatus described previously (Kyle et al., 1984; Schuster et al., 1988).

RESULTS

Induction of ELIP in Mature Plants Exposed to Light Stress—Mature pea plants grown at normal light regime (12 h light/12 h dark) at a light intensity of 40 μE/m²s do not have detectable levels of ELIP mRNA as assayed by dot blot hybridization and in vitro translation of poly(A')-RNA. Also the corresponding protein cannot be detected (Fig. 1). However, when such plants are exposed to high light intensity the level of ELIP mRNA rises considerably as demonstrated by dot blot hybridization (Fig. 1A), in vitro translation of poly(A')-RNA (Fig. 1B), and immunoprecipitation of the ELIP precursor from the translation products (Fig. 1C). The translation product accumulates in vivo in the thylakoid frac-

![Fig. 1. Induction of ELIP transcript and accumulation of protein in mature pea leaves during light stress. Detached pea leaves were exposed to high light (2,500 μE/m²s) for 0 or 2 h, and the changes in mRNA and protein levels were analyzed. A, dot blot hybridization of isolated poly(A')-RNA with a labeled insert of ELIP clone; B, in vitro translation of poly(A')-RNA followed by autoradiography. Arrows indicate the position of precursors, pELIP, pLHCP, and pSSU (small subunit of ribulose-1,5-bisphosphate carboxylase); C, immunoprecipitation of pELIP from the translation mixture of B; D, pulse labeling of proteins with [35S]methionine followed by SDS-PAGE and autoradiography; E, Western blot.](image1)

![Fig. 2. Accumulation of ELIP in pea leaves of different ages during high light treatment. Detached leaves of increasing age (marked 1–5) from a 14-day-old plants were floated on water in the presence of [35S]methionine (100 μCi/ml) and exposed to high light (3,000 μE/m²s) for 2 h. Chloroplast membranes were isolated and proteins were separated by SDS-PAGE, A, autoradiogram; B, Western blot. The larger leaf (2) is the second one and it seems only to emerge above leaf 1, due to the orientation of the petiole when the plant was laying on the support.](image2)

![Dot Blot Hybridization-Insert of an ELIP cDNA clone (Scharnho et al., 1985) was labeled using random oligonucleotides as primers according to Boehringer Mannheim protocol. Dot blot hybridization was performed as described before (Otto et al., 1988).](image3)
plants grown in the light for 14 days were detached, floated on water in the presence of [³⁵S]methionine, and exposed to strong light (3,000 μE/m²s) for 2 h. The radioactive-labeled ELIP was detected by autoradiography (Fig. 2A). The identification of the ELIP band indicated in the autoradiogram was verified by Western blotting (Fig. 2B). ELIP was synthesized in significant amounts in all leaves except the oldest (leaf 5) which was already at the beginning of the senescence process and in which only traces of ELIP were present. The level of ELIP was higher in the second but very similar in leaves 1, 3, and 4. This mode of ELIP expression is very similar to that of D1 protein, while expression of LHCP declines much faster with the developmental age of leaves than that of ELIP.

The ELIP mRNA was shown to be induced only transiently in etiolated plants during the first 2–4 h of the greening process (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987). To establish whether this is the case also in the mature leaves in which ELIP was induced by light stress, the amounts of ELIP mRNA were estimated as a function of time after transferring the leaves from high to low light (recovery) conditions. The results of such an experiment demonstrate that ELIP mRNA is short-lived as compared with the mRNA of LHCP or small subunit of ribulose-1,5-bisphosphate carboxylase (Fig. 3, A and B). The half-lifetime of ELIP mRNA is about 1 h under the above experimental conditions as demonstrated by both dot blot hybridization (Fig. 3A) as well as in vitro translation and immunoprecipitation of ELIP precursor from the translation products (Fig. 3, B and C).

**Light Intensity Dependence of ELIP Induction and Degradation Are Compatible with the Photoactivation and Recovery Processes**—Plants exposed to strong light lose PSII activity as a function of light intensity and time of exposure (Kyle et al., 1984; Prasil et al., 1992). Previous results demonstrated that the amount of ELIP mRNA induced is proportional to the light intensity (Adamska et al., 1992b). Thus, it was of interest to test whether a correlation exists between these phenomena and the accumulation of ELIP at high light intensity.

Detached leaves of 14-day-old light grown pea plants were exposed for 3 h to white light of increasing intensities, and the degree of photoinhibition (Fig. 4A) and ELIP accumulation in the thylakoid membranes (Fig. 4B) were assayed. Photosystem II activity, estimated by measurements of the variable fluorescence ($F_v$) was gradually lost as a function of light intensity. The activity could be recovered if the leaves were transferred to low light condition for an additional period of 4 h. However, the degree of recovery decreased with the increasing extent of photoinhibition. As shown in Fig. 4B, small amounts of ELIP could be detected already in thylakoids of leaves exposed to 500 μE/m²s which have lost about 30% of their PSII activity. A gradual increase in ELIP level was observed as the light intensity increased from 500 to 3,000 μE/m²s, and this corresponded with the gradual increase in PSII photoactivation.

It is well established that both the synthesis and degradation (turnover) of the reaction center II, D1 protein increase under light stress conditions. Thus it was of interest to determine whether a similar situation occurs also for ELIP. For this purpose, detached pea leaves were labeled with [³⁵S]methionine at a light intensity of 3,000 μE/m²s for 2 h and then transferred to fresh medium containing unlabeled methionine and incubated at the same light intensity for an additional 8 h. The results of this experiment (Fig. 5) show that indeed the D1 protein is rapidly synthesized during the pulse labeling and degraded during the chase period in high light exposed leaves as expected (Prasil et al., 1992). However, ELIP was not degraded under these conditions (Fig. 5). As opposed to its stability during high light treatment ELIP is degraded when the leaves are transferred to recovery conditions at low light intensity and resume their photosynthetic activity. However, the rate of ELIP degradation shows a very peculiar pattern as indicated in Fig. 6A. The amount of ELIP degraded under recovery conditions decreases in an inverse function of the extent of the initial light stress. ELIP induced at 500 μE/m²s is almost completely degraded within 1–2 h during recovery at 40 μE/m²s, while ELIP induced at 3,000 μE/m²s shows no significant degradation for up to 4 h under the same recovery conditions (Fig. 6A). In leaves exposed to alternating high and low light for periods of 2 h, a significant loss of ELIP can be detected during the low light exposure if the light intensity used during the induction period was 1,000 μE/m²s but not if the induction was carried out at 2,000 μE/
Onine during high light treatment then chased at the same light intensity for different times as indicated. The thylakoid membranes were isolated, and their proteins were separated by SDS-PAGE followed by autoradiography. D1, LHCP, mature leaves. The thylakoid membranes were isolated, and their proteins were separated by SDS-PAGE followed by Western blotting. ELIP were identified by Western blotting of thylakoid proteins resolved by SDS-PAGE indicated that ELIP level induced by the strong light was maintained in leaves exposed to lower light intensities down to 500 μE/m²s. The level of ELIP decreased slightly in leaves kept at 200 μE/m²s and was almost undetectable in leaves transferred to 40 μE/m²s (Fig. 7). Induction of ELIP mRNA does not occur at light levels below 400 μE/m²s (Adamska et al., 1992b). ELIP mRNA is short-lived and is continuously degraded even under conditions which sustain its accumulation; this was proven by experiments in which preilluminated leaves were treated with amounts of actinomycin D sufficient to inhibit transcription (data not shown). Therefore, these data indicate that ELIP is stable at all light intensities above those causing its induction.

**ELIP Accumulation Is Enhanced by Treatment of Leaves with Bleaching Herbicides**—It has been reported previously that light stress affecting PSII induces an accelerated synthesis of carotenoids and interconversion to xanthophylls. This phenomenon may relate to protection of PSII against light-induced free radicals (Demmig et al., 1987; Demmig-Adams, 1990). We have previously shown that the bleaching herbicide fluridone does not affect the synthesis of ELIP mRNA induced by strong light but enhances somewhat the level of the accumulated protein (Adamska et al., 1992a). Bleaching herbicides may cause degradation of chlorophyll-binding proteins in high light-exposed leaves and thus could increase the level of free chlorophyll which may be harmful upon light sensitization. Thus one could consider also the possibility that ELIP may be involved in binding free chlorophyll and ascribe protection from free radical formation. To explore this possibility pea leaves from 14-day-old light-grown plants were exposed for 6 h to light stress in the presence or absence of various bleaching herbicides, and changes in the pigment and ELIP contents were assayed as a function of time. The results of this experiment (Fig. 8) show a remarkable parallel in the inhibition of carotenoid synthesis induced by high light and in the accumulation of ELIP. Inhibition of carotene synthesis by fluridone acting as an inhibitor of the phytoene desaturase which converts phytoene into phytofluene and carotene (Chamovitz et al., 1990) results in a drastic reduction of the zeaxanthin level without significant effect on the level of chlorophyll (Fig. 8B). Norflurazon, a noncompetitive inhibitor (Simpson et al., 1986; Sagar et al., 1988) acting at the same time. Detached leaves were pulse-labeled with [35S]methionine during high light treatment (HL, 3,000 μE/m²s) for 2 h and then chased at the same light intensity for different times as indicated. The thylakoid membranes were isolated, and their proteins were separated by SDS-PAGE followed by autoradiography. D1, LHCP, and ELIP (arrows) were identified by Western blotting.

**Fig. 6.** Kinetics of ELIP degradation during recovery from light stress. Detached leaves were illuminated for 2 h with different high light intensities (HL) as indicated in the figure and then transferred to low light conditions (Recovery or LL, 40 μE/m²s) for 20 h (A) or alternatively exposed to cycles of high light (HL) of 2 h followed by exposure to low light (LL) for 2 h (B). Samples were taken before (lanes C in both panels) or after light treatments for times as indicated, chloroplast membranes were isolated and their proteins were separated by SDS-PAGE followed by Western blotting.

m²s. In this case a higher level of ELIP is induced, and a decrease is observed only during the first low light exposure but not during the next consecutive cycle. Thus, the detected level of ELIP seems to be constant in this case (Fig. 6B).

Since ELIP was found to be stable at high light intensities, the question arose as to whether its degradation occurs gradually as a function of lowering the light intensity. To answer this question detached pea leaves were exposed to 1,500 μE/m²s for 2 h, and then the leaves were further incubated at various light intensities for an additional 4-h period. Western blotting of thylakoid proteins resolved by SDS-PAGE indicated that ELIP level induced by the strong light was maintained in leaves exposed to lower light intensities down to 500 μE/m²s. The level of ELIP decreased slightly in leaves kept at 200 μE/m²s and was almost undetectable in leaves transferred to 40 μE/m²s (Fig. 7). Induction of ELIP mRNA does not occur at light levels below 400 μE/m²s (Adamska et al., 1992b). ELIP mRNA is short-lived and is continuously degraded even under conditions which sustain its accumulation; this was proven by experiments in which preilluminated leaves were treated with amounts of actinomycin D sufficient to inhibit transcription (data not shown). Therefore, these data indicate that ELIP is stable at all light intensities above those causing its induction.

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**Fig. 5.** Stability of ELIP during high light treatment of mature leaves. Detached leaves were pulse-labeled with [35S]methionine during high light treatment (HL, 3,000 μE/m²s) for 2 h and then chased at the same light intensity for different times as indicated. The thylakoid membranes were isolated, and their proteins were separated by SDS-PAGE followed by autoradiography. D1, LHCP, and ELIP (arrows) were identified by Western blotting.

**Fig. 7.** Degradation of ELIP induced by high light in leaves further exposed to decreasing light intensities. Detached leaves were exposed to high light (HL, 1,500 μE/m²s) for 2 h and then transferred to decreasing light intensities for different times. Thylakoid membranes were isolated and their proteins separated by SDS-PAGE followed by Western blotting.
FIG. 8. Relation between accumulation of ELIP and loss of pigments in leaves exposed to light stress in presence or absence of carotenoid biosynthesis inhibitors. Detached leaves were exposed to high light (3,000 μE/m²s) for different times in the absence (A) or the presence of 20 μM fluridone (B), 100 μM norflurazon (C), or 10 μM pyrimidine analog KM 145-155 (D). The pigments and chloroplast membranes were isolated and analyzed by HPLC or Western blotting, respectively. The 100% values were those of the zero time and were compared on a leaf wet weight basis.

The site has a somewhat higher effect on the level of zeaxanthin; in its presence a significant reduction is observed also in the level of β-carotene (Fig. 8C). With both inhibitors a 2–3-fold higher accumulation of ELIP is evident as compared with the ELIP level in thylakoids of leaves illuminated in absence of these herbicides (compare Figs. 8, B and C with A). The highest ELIP induction is, however, obtained in leaves exposed to strong light in presence of the inhibitor KM-145-155 which blocks ζ-carotene desaturase and promotes the accumulation of ζ-carotene (Chollet et al., 1990). In this case an enhanced loss of both chlorophylls and carotenoids is observed. However, the kinetics of pigment changes do not coincide in time with the increase in the ELIP content neither for the increase in lutein and zeaxanthin (Fig. 8, A–C) nor for the loss of zeaxanthin or chlorophyll a and b (Fig. 8, C and D). In all cases a major change in the respective pigments has already occurred after 2 h of exposure to the strong light in presence of the various herbicides, while a significant increase in the ELIP content is noticed only after 2 h.

DISCUSSION

The results presented in this work demonstrate that ELIP synthesis is related to the light stress response of pea plants. Light intensity controls ELIP expression at both levels, the transcription, and the accumulation of the protein. The light effect is cumulative. Higher levels of response are induced by higher light intensities up to a saturation level attained at light intensities compatible with extensive light-induced inactivation of PSII. However, the circadian expression of ELIP transcript in green plantlets (Otto et al., 1988) which occurs daily before the transition from dark to light indicates an
existence of additional circadian-regulated control. Previous results have indicated that blue light (410–480 nm) and UV-A light (355–365 nm) are responsible for the induction of ELIP mRNA (Adamska et al., 1992a, 1992b). The blue light effect in the activation of ELIP transcription can be also related to that of carotenoid synthesis which has been found to be blue light controlled in Phycomyces (Rau, 1930) and Neurospora (Baima et al., 1991).

The major finding of this work is the fact that ELIP does not significantly turn over in strong light, its degradation being facilitated only when the light intensity is reduced to low levels which do not support ELIP induction. This would indicate that in nature once induced, ELIP will persist for extended periods of time throughout the day and will be lost only during the late part of the day and during the night. The persistence of ELIP in high light treated leaves even when the light intensity is reduced to low levels may be correlated with its assumed protective role against photo-inhibition and degradation of reaction center II proteins (Adamska et al., 1992b). The data presented in Fig. 4 are in agreement with this interpretation. Above a light intensity of 2,000 μE/m²s recovery from photo-inhibition is rather slow; under these conditions accumulation of ELIP is the highest, and, in addition, the protein is stabilized and its degradation proceeds very slowly (Fig. 6). However, the mechanism of ELIP action remains still obscure.

One possible role of ELIP could be its involvement in the light-induced changes in the pigment content and/or composition in the photosynthetic membranes. The enhanced accumulation of ELIP in the presence of inhibitors blocking carotenogenesis could be considered as an indication for a relation of ELIP with one of these processes. However, the increase in the ELIP level induced by bleaching herbicides neither coincides in time with the changes in the levels of individual carotenoids nor with the fast degradation of chlorophylls. A good correlation exists, however, with the intensity of the light stress which is higher for bleached plants than for control plants with normal pigment content. One should keep in mind that these extreme stress conditions induced by strong light and bleaching herbicides might represent a situation which is far beyond the capability of the system to respond promptly to this artificial combination of stresses.

Previous results (Demmig-Adams, 1990) have demonstrated that the xanthophyll cycle is activated by exposure of plants to high light intensities. The activation of this cycle resulting in the interconversion of violaxanthin via antheraxanthin to zeaxanthin and that of β-carotene to zeaxanthin may alleviate the light stress effect by a 2-fold action: increase in the nonradiative energy dissipation by the antennae system of photosystem II and scavenging of free radicals or active oxygen species resulting from their interaction with oxygen (Demmig et al., 1987; Demmig-Adams, 1999; Prasil et al., 1992). While the first mechanism is controversial (Havaux et al., 1991) the second seems to be generally accepted. Upon reduction of light intensity, zeaxanthin is converted back to violaxanthin (Bilger et al., 1989; Demmig-Adams, 1990). The time scale of ELIP accumulation, the threshold light intensity required for ELIP synthesis, and accumulation with those required for the xanthophyll cycle activation represent reasonable correlations and as such support the concept that ELIP may be involved in this process. Inhibitors of phytoene desaturase and β-carotene desaturase lead to an accumulation of phytoene or β-carotene, respectively, and consequently to a reduction of β-carotene levels. The lack of carotenoids and increased level of their intermediates may interact with ELIP translation system as a reaction of the cell trying in this way to overcome the block in the carotenogenesis pathway arrested by herbicides. The fact that under these conditions an excessive amount of ELIP accumulates could be considered as proof of the postulated hypothesis. In this context, it is of interest that an ELIP-like protein was found to be induced in the unicellular alga Dunaliella bardawil which responds to high light intensity by extensive β-carotene accumulation (Lers et al., 1991).

However, it is possible that light stress conditions induces parallel cellular responses which may coincide in time and may be elicited by the same stress conditions but have independent functions. Light stress results also in degradation of reaction center proteins and release of pigments. Free chlorophyll may be harmful to its environment when sensitized by light absorption. Thus, it is possible that ELIP may have a function as a chlorophyll scavenger. This possibility should be considered in view of the fact that cross-linking experiments indicate that under high light conditions ELIP interacts with the D1 protein of PSII reaction center (Adamska and Kloppstech, 1991) whose degradation in enhanced under these conditions. Other possibilities can also be envisaged and thus the function of ELIP is still under investigation.

Originally it had been assumed that ELIPs are confined to early stages of plastid development (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987). In the present paper (Fig. 2) we show that ELIP induction is obtained under high light conditions even in mature green leaves. One has to consider that the etiolated or young emerging plants in which ELIP can be induced at relatively low light intensity are actually exposed to light stress, since the pigment content (chlorophylls, carotenenes, and xanthophylls) of such plants is extremely low as compared to that of the mature plants used in the present work. In this respect we consider ELIP as being a light-stress response protein in the etiolated, developing as well as in mature, fully developed plants.

Acknowledgments—We are grateful to Dr. R. Neechusthai (Jerusalem), for reading this manuscript, to Prof. J. Hirschberg (Jerusalem), for his kind gift of fluoridine and norfluoradazol, to Prof. P. Böger (Graz, Austria), for sending us the pyrimidine derivative KM 145-155, and to Prof. F. Herzfeld (Hannover), for the anti-ELIP antibodies. We also thank J. Beator (Hannover) and D. Chavovitz (Jerusalem), for their help with the HPLC analysis and T. Mor and Dr. H. Zet (Jerusalem) for helping with computer graphic programs.

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Light Stress Regulates ELIP Stability

5443
Light Stress Regulates ELIP Stability