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Expression of Early Light-Inducible Proteins in Flag Leaves of Field-Grown Barley[1]

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Early light-inducible protein (ELIP) mRNA and protein levels were analyzed during maturation and senescence of barley (*Hordeum vulgare* L.) flag leaves under field conditions. The data clearly demonstrate that ELIP mRNA levels are related to the sunlight intensity before sample collection. Levels of mRNAs encoding both low and high molecular mass ELIPs fluctuate in parallel. Changes in mRNA levels are accompanied by corresponding changes in protein levels except for days when average temperatures are high. Comparison of flag leaves at different stages of development in spring and winter barley varieties suggests that light-stress-regulated ELIP gene expression is independent of the developmental stage of the leaves. Although chlorophyll content, photosystem II (PSII) efficiency, and 32-kD herbicide-binding protein of PSII levels decrease drastically after the onset of senescence, ELIP mRNA and protein still accumulate to high levels on bright days.

ELIPs were first discovered in etiolated developing plants during the first hours of the greening process (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987). Recent investigations with pea (Adamska et al., 1992a, 1992b) and barley (*Hordeum vulgare* L., Pötter and Kloppstech, 1993) show that ELIPs are also expressed in mature green plants under light-stress conditions.

Barley plastids contain two different families of ELIP: high molecular mass proteins of 18 to 18.5 kD and low molecular mass proteins of 13.5 kD (Grimm and Kloppstech, 1987). These are encoded by two small gene families that are distinguished by the presence or absence of a 25- to 28-amino acid peptide at the amino-terminal end of the mature protein (Grimm et al., 1989; Kruse and Kloppstech, 1992). No differences in their expression patterns have been reported until now.

The function of ELIP proteins is still not understood. Under certain light-stress conditions in pea leaves, an increase in the level of ELIP parallels a decrease in the amount of the D1 protein of PSII (Adamska et al., 1992b), suggesting an interrelationship of the two phenomena. A possibility is that ELIPs play a role in the stabilization and insertion of newly synthesized D1 during repair of photodamaged PSII centers (Pötter and Kloppstech, 1993). Cross-linking experiments indeed showed the proximity of ELIP to the D1 protein of PSII (Adamska and Kloppstech, 1991). The results of ELIP expression are also consistent with an involvement of ELIP in pigment synthesis and organization. Proteins similar to ELIPs and correlated with the overexpression of carotenoids have been found in the green alga *Dunaliella* (Lers et al., 1991). Therefore, it is possible that ELIP acts as a carrier for carotenoids that have a protective function in pigment protein complexes of the photosynthetic apparatus (Adamska and Kloppstech, 1994). In this context an involvement of ELIPs in the xanthophyll cycle, a protective mechanism against photoinhibition (Demmig-Adams, 1990), is considered (Levy et al., 1993).

In this paper ELIP expression was analyzed in flag leaves of field-grown barley. The results show that under field conditions ELIP expression is related to light intensity. Although PSII efficiency and D1 levels decline with advancing senescence of the leaves, ELIP expression appears to be independent of the developmental stage of the leaves.

MATERIALS AND METHODS

Plant Material

Spring barley (*Hordeum vulgare* L., cv Carina) and winter barley (*Hordeum vulgare* L., cv Trixi) were grown in 1992 under agricultural conditions in a field (0.5 ha) of the Institut für Angewandte Botanik (Universität Hamburg) situated in Wulfsdorf, near Hamburg. Flag leaves were collected and analyzed every other day beginning June 15 and ending July 7. A similar experiment had been performed in 1991 with the spring variety of barley alone. Sample collections and measurements were always made between 10 and 12 AM. Samples were either used directly for measurements of fluorescence and Chl content or immediately frozen and transported to the laboratory in liquid nitrogen and then stored at −80°C.

Meteorological Measurements

To monitor temperature and irradiance, a meteorological station with a data-logging device (LI-1000; Li-Cor, Walz, Effeltrich, Germany) was established in the field. Tempera-

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Abbreviations: D1, 32-kD herbicide-binding protein of PSII; ELIP, early light-inducible protein; F_m, maximal fluorescence yield; F_o, nonvariable fluorescence yield; F_v, variable fluorescence yield; PFD, photon fluence density.

ture (°C) of air and soil as well as PFDs (μE m^{-2} s^{-1}) were measured every minute. The average of these measurements was then recorded every 2 h beginning at 1 AM (Central Europe Time) each day. The determination of mol of photons per m^2 measured in the field during the 12-h prior to leaf collection was by integration of all PFD data measured during this time.

Chl Fluorescence Measurements

Chl fluorescence parameters were measured at room temperature using a pulse amplitude modulation fluorometer (Walz, Effeltrich, Germany) according to the instructions given by Schreiber et al. (1986). Measurements were performed with intact flag leaves at a position 1.5 cm above the ligule and at a position 1.5 cm below the leaf tip. For each measurement, 15 different leaves were used. Mean values are based on the average of 15 basal and 15 apical segments. Prior to fluorescence measurements, the leaf blades were dark adapted for 30 min. The yield of F_o was measured with weakly modulated (1.6 kHz) light of approximately 0.1 μmol m^{-2} s^{-1}. This intensity is sufficiently low not to produce any significant F_v. F_m was determined by application of a saturating flash of white light (4000 μmol m^{-2} s^{-1}) of 700 ms duration. The F_v/F_m ratio, which is a measure of the quantum yield of the photochemistry of PSII centers, was determined as $(F_m - F_o)/F_m$ (Butler, 1977).

Analysis of Chl Content

Chl's were extracted from different flag leaf blades with hot methanol and their concentrations were determined spectrophotometrically using the formulas given by Holden (1965). Three independent measurements were done for each determination.

Isolation and Analysis of RNA

Total RNA of barley flag leaf blades was isolated as described (Chirgwin et al., 1979) and fractionated on 1% (w/v) agarose gels containing formaldehyde. The RNA was then transferred by capillary blotting onto positively charged nylon membranes (Zeta Probe; Bio-Rad, München, Germany). The membranes were prehybridized at 42°C for 5 min in a solution consisting of 50% (v/v) deionized formamide, 0.25 M sodium hydrogen phosphate, pH 7.2, 0.25 M sodium chloride, 1 mM EDTA, and 7% (w/v) SDS. Hybridization was carried out overnight at 42°C in the same solution after addition of the radiolabeled probe. After the filters were washed, they were autoradiographed using Kodak X-OMAT, AR films (Eastman Kodak, Rochester, NY).

Radiolabeled probes were prepared by random primed labeling with [α-^{32}P]dCTP using a kit (Boehringer, Mannheim, Germany). The homologous cDNA insertions of pHV58 and pHV60, specific for high and low molecular mass ELIP proteins (Grimm and Kloppstech, 1987), respectively, were used for detection of ELIP mRNA. For analysis of mRNA levels specific for ACC synthase, a 340-bp fragment amplified from tomato total DNA by PCR with specific primers (T. Kleber-Janke, unpublished result) was used. Densitometric analysis of the autoradiograms was performed using video 1D-BAsys equipment (Biotec Fischer, Reiskirchen, Germany). Signals were corrected for background density.

Immunological Analysis of ELIP Protein Levels

Total proteins were extracted from frozen leaf blades as described by Pötter and Kloppstech (1993). Protein concentrations were determined with a TCA-precipitated aliquot by the method of Lowry et al. (1951). Equal amounts of proteins (20 μg) were subjected to SDS-PAGE (Fling and Gregerson, 1986). Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA) by electroblotting with a semidry transfer apparatus (Biotec Fischer) at 4°C with a transfer buffer consisting of 20 mM Tris and 150 mM Gly. Before the gel was blotted, it was presoaked in transfer buffer including 0.1% (w/v) SDS, and the membrane was adjusted to transfer buffer containing 30% (v/v) methanol. After the sample was incubated with the primary antibody directed toward barley low molecular mass ELIP (Pötter and Kloppstech, 1993), immunoreactive protein bands were visualized using a peroxidase-coupled anti-rabbit serum with chemiluminescence detection (Amersham, Braunschweig, Germany).

RESULTS

Maturation and Senescence of Barley Flag Leaves

During the field experiment in 1992, flag leaves of spring barley (cv Carina) reached their final lengths on June 19 when they exhibited the maximum value for F_v/F_m (0.8) (Fig. 1). Chl content was highest 2 d later in leaves collected on June 21. Thereafter, Chl content changed little until June 29

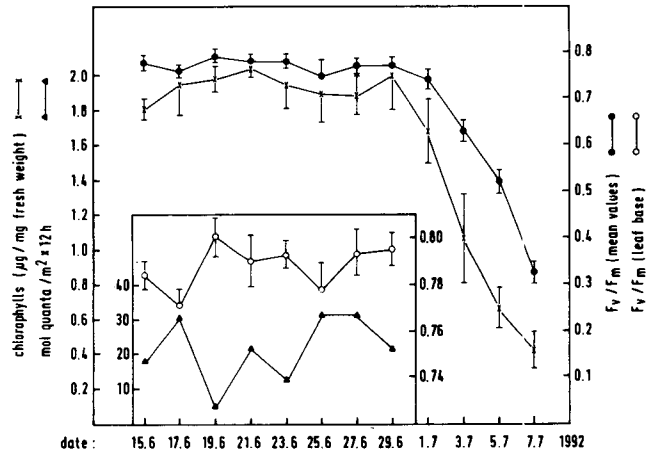

Figure 1. Chl contents and F_v/F_m ratios (mean values from basal and apical sections) (●) of flag leaves of the spring barley cv Carina collected every other day from June 15 until July 7, 1992, in a field. Inset, The course of F_v/F_m values measured at the bases of the leaves (○) from June 15 until June 29 is compared to light intensities (mol quanta/m^2) measured during the last 12 h before sample collection. Data are mean values ± SD from 3 (Chl) or 15 (fluorescence) independent measurements.

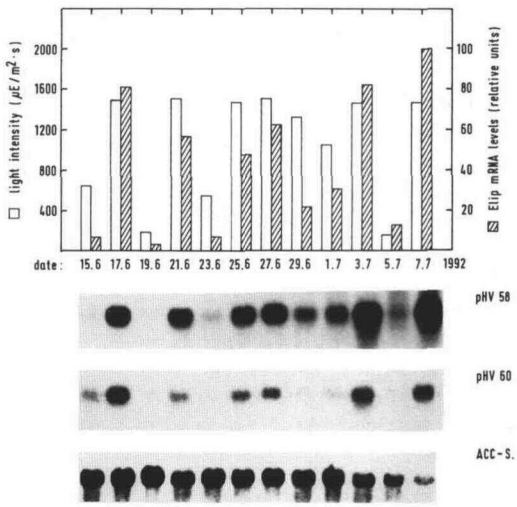

Figure 2. Northern blot analysis of ELIP mRNA levels in flag leaves of spring barley cv Carina collected from June 15 until July 7, 1992. Hybridization was subsequently carried out with the insertion of the two cDNAs, pHV58 and pHV60, specific for high and low molecular mass ELIPs, respectively, as probes. For comparison, hybridization was carried out with a probe specific for ACC synthase mRNA. Signals on the autoradiogram (pHV58) were densitometrically quantified and compared to light intensities (μE m^{-2} s^{-1}) measured prior to sample collection (see "Materials and Methods") (top of figure).

(Fig. 1). After this day Chl content and F_v/F_m values decreased in parallel, indicating the onset of senescence processes on July 1 at the latest. The slight fluctuations in F_v/F_m observed during the time before the onset of senescence were more pronounced at the leaf base and showed an inverse correlation to the photon fluence measured in the field during the last 12 h prior to sample collection (Fig. 1, inset), indicating a possible modulation of PSII efficiency (Krause and Weis, 1991). An inverse relationship between fluorescence levels and light intensities was observed previously in a similar experiment in 1991 (data not shown). After the onset of senescence, changes in light intensity did not appear to affect Chl fluorescence.

Fluctuations in ELIP mRNA Levels in Relation to Sunlight Intensity

Total RNA was extracted from 12 different leaf blade samples of spring barley (cv Carina), similarly to those used for the physiological measurements. ELIP mRNA levels were analyzed by northern blot hybridization using the insertions of the cDNA clones pHV58 and pHV60 as probes (Grimm and Kloppstech, 1987). The autoradiograms show dramatic fluctuations of the mRNA levels among the various samples (Fig. 2). Levels of mRNAs, specific for both the high and low molecular mass ELIPs, changed in parallel. For comparison, levels of mRNA specific for ACC synthase involved in ethylene biosynthesis are also shown (Fig. 2). Unlike the ELIP transcripts, this mRNA accumulated steadily in flag leaves before July 3 and then decreased slightly due to senescence.

As shown in Figure 2, ELIP mRNA levels correlated with the sunlight intensities measured in the field prior to sample collection. On days with high light intensities, e.g. June 17 or June 21, there were high levels of ELIP mRNA, and on days with low sunlight intensities, e.g. June 15, June 19, or June 23, only low levels of ELIP mRNA were detected. To analyze this correlation in more detail, ELIP mRNA levels determined in flag leaves of field-grown winter barley cv Trixi (1992) and summer barley cv Carina (1991 and 1992) on various days during maturation and senescence were plotted against light intensities measured prior to sample collection in each case (Fig. 3). The regression analysis revealed an exponential curve; on days with light intensities of less than 700 μE m^{-2} s^{-1}, ELIP mRNA was hardly detectable, but beyond this threshold, increasing light intensity resulted in a drastic increase in ELIP mRNA. In Figure 3 closed circles indicate days with average temperatures exceeding 22°C in the field. On these days there was no correlation between ELIP mRNA and light intensity, indicating that the usual response to light was diminished under heat-stress conditions (cf. Howard and Ougham, 1993).

ELIP mRNA Is Expressed in Both Young and Senescing Flag Leaves

As shown in Figure 2, ELIP mRNA levels obviously did not depend on the developmental stage of the leaves. For example, in comparison to leaves that were collected on June 17, those collected on July 7 were 20 d older and had only 20% of the original Chl content (Fig. 1), but both samples had similar levels of ELIP mRNA.

Figure 4 shows a comparison between ELIP mRNA levels in flag leaves of summer barley cv Carina and winter barley cv Trixi. The leaves of summer barley and winter barley were

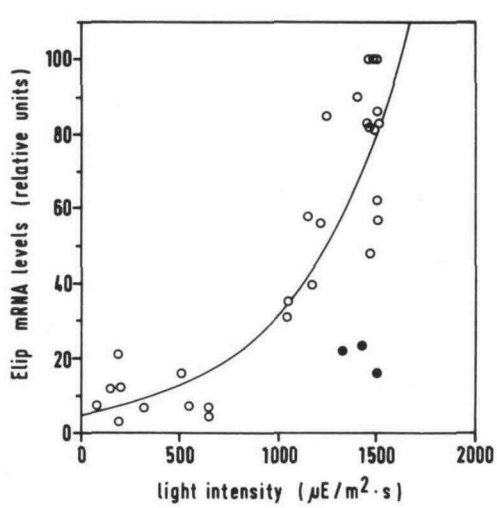

Figure 3. Relationship between ELIP mRNA levels of field-grown barley flag leaves and light intensities measured prior to sample collection. ELIP mRNA levels in flag leaf samples collected on various days during maturation and senescence of summer barley cv Carina (1991 and 1992) and winter barley cv Trixi (1992) were analyzed by northern blots as described in Figure 2. The best fit curve is $y = 4.909 \times 1.002x$; $r = 0.89$, $n = 29$. ●, Data determined on days with an average daily temperature greater than 22°C.

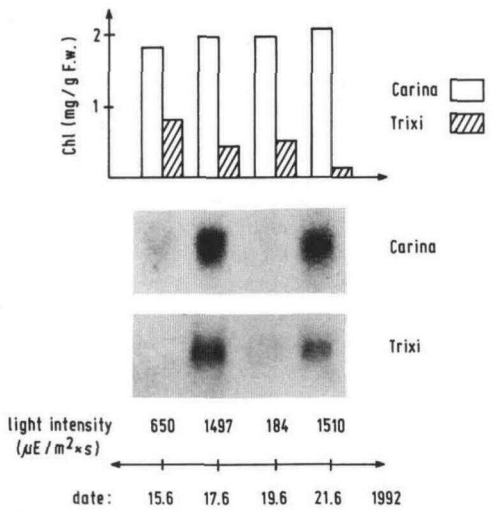

Figure 4. Northern blot analysis of ELIP mRNA levels in flag leaves collected on June 15, 17, 19, and 21 in a field of winter barley cv Trixi and a field of spring barley cv Carina. The insertion of the cDNA clone pHV58 was used as a probe. The Chl contents of leaves derived from the winter barley field and the spring barley field are shown in the top part of the figure. Samples were collected at the same times.

collected at exactly the same time of the day. Both fields were situated close to each other, and hence, the environmental conditions experienced by the leaves must have been very similar, whereas the developmental stages of the leaves differed considerably between the varieties. The flag leaves derived from the winter variety were fully developed on May 26 and were already in the process of senescence when samples were collected. In contrast, leaves of the spring barley reached their final length on June 19. As a consequence, the Chl contents of the leaves of winter barley were much lower than those of spring barley. In spite of these differences, leaves of both varieties showed parallel fluctuations in ELIP mRNA levels as determined by hybridization with the pHV58 probe in northern blots (Fig. 4).

ELIP and D1 Protein Levels

ELIP protein levels were analyzed in 12 spring barley flag leaf samples collected at the same times as those samples that had been used for analysis of mRNA levels. Western blot analysis was performed with an antibody specific for the low molecular mass group of ELIPs (Pötter and Kloppstech, 1993). The immunological reaction revealed distinct fluctuations in ELIP protein levels during the time of sample collection (Fig. 5). In most cases these fluctuations occurred in parallel to variations in the mRNA levels; however, some exceptions were obvious. Although ELIP mRNA was clearly detectable on June 25 and June 27 (Fig. 2), ELIP proteins did not accumulate on these days (Fig. 5). The days from June 25 until July 1 were very hot with average day temperatures greater than 20°C. We assume that the high temperature interfered with ELIP gene expression. Measurements of ELIP levels in relation to temperature, carried out in the laboratory, indeed showed that ELIP gene expression in barley decreased at high temperatures (O. Stoysel, unpublished results).

To determine whether ELIP accumulation was interrelated with D1 degradation as suggested previously (Adamska et al., 1992b), the same protein samples were analyzed immunologically with an antibody specific for the D1 protein (Johanningmeier, 1987). However, D1 levels did not show inverse fluctuations to ELIP protein levels or parallel changes to F_v/F_m values (Fig. 5). Whereas D1 levels stayed almost constant in young flag leaves, they drastically declined in later stages of leaf development. Longer exposures of immunochemically probed western blots showed that D1 protein was still present in leaves collected between June 29 and July 7 (data not shown). The levels of D1 protein during this period were, however, much lower than the levels shown before June 29.

DISCUSSION

The levels of ELIP mRNA in flag leaves of field-grown barley are correlated with the average PFD received by the leaves before sample collection. The threshold light intensity of about 700 $\mu E\ m^{-2}\ s^{-1}$ for induction of ELIPs is of the same order of magnitude as has been reported for barley primary foliage leaves collected from plants grown under controlled environmental conditions in the laboratory (Pötter and Kloppstech, 1993).

Although it could be expected that in the fields different combinations of environmental factors might have an influence on expression of the two types of barley ELIPs, we observed that the fluctuations in mRNA for high molecular mass ELIPs closely parallel the fluctuations in mRNA levels of low molecular mass ELIPs. Levels of ELIP mRNA and protein, however, do not always change in parallel. On several days low levels of mRNAs, but no proteins, are detectable. High temperatures during the days when ELIP proteins are not accumulating could be responsible for this discrepancy. This finding agrees with earlier data, which indicate that ELIP expression is controlled at both transcription and translation levels (Pötter and Kloppstech, 1993; Adamska and Kloppstech, 1994).

Under natural field conditions ELIP expression seems to be largely independent of the developmental stage of the leaves. Developing, mature, and senescing leaves obviously have the same capacity to synthesize ELIP in response to light intensity. This result may be surprising considering the dramatic loss of proteins during leaf senescence (Brady, 1988). However, proteins are lost mainly by an activated degradation

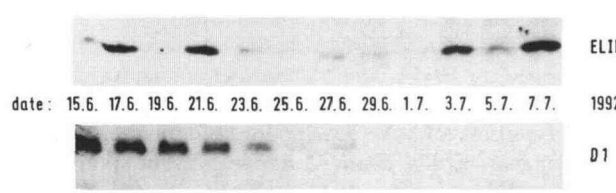

Figure 5. Western blot analysis of ELIP and D1 protein levels in flag leaves collected from the spring barley cv Carina field from June 15 until July 7. The samples were identical with those used before for analysis of mRNA levels (cf. Fig. 2).

process during senescence and to a lesser extent by a decrease in protein synthetic capacity (Lamattina et al., 1985). It seems that the senescence-specific increase in proteolytic activities does not affect ELIP protein levels. A comparison of ELIP expression in leaves sampled at the same time from fields of different barley varieties at different stages of development emphasizes ELIP's primary relation to light intensity and its relative independence of other factors, such as developmental stage of the leaves and corresponding Chl contents.

In the period before onset of senescence, changes in light intensity seem also to affect PSII efficiency. The slight variations in F_v/F_m ratios are accompanied by similar variations in the oxygen evolution rates (data not shown) and show an inverse relationship to the sum of photons received during the last 12 h before leaf collection. Therefore, prior to the onset of senescence, high ELIP levels usually coincide with low values of F_v/F_m. This might indicate a protective function of ELIP against damage of the photosynthetic apparatus during light stress (cf. Adamska and Kloppstech, 1994). One major protective mechanism against photoinhibition is the turnover of the D1 protein. Adamska et al. (1992b) reported a close relationship between ELIP expression and D1 breakdown under light-stress conditions. In leaves of field-grown barley, however, no inverse fluctuations were detectable between D1 and ELIP levels. The D1 level stays almost constant in young flag leaves and drastically declines in later stages of leaf development. However, high levels of ELIPs can be observed even in leaves collected during the 1st week of July when almost no D1 protein is left. We interpret the early decrease in D1 levels as an indication of the first phase of the breakdown of PSII associated with senescence. Interestingly, photosynthetic capacity of flag leaves measured as oxygen evolution rates at high saturating light intensities decreases in parallel to the D1 level starting on June 23 (data not shown).

According to our data ELIP does not seem to play a role in the repair of D1 but is more likely to be involved in a different mechanism protecting plastids against light stress. Such a mechanism would be useful also for photosynthetically competent chloroplasts and senescent chloroplasts. The results support the idea that ELIP acts as a pigment carrier or scavenger. These pigments could be carotenoids having a protective function under light-stress conditions or Chl's either during their synthesis and the insertion of the pigment protein complexes into the membrane or during their senescence-associated degradation. The protection of pigments by binding to ELIP could be especially important during the reorganization of pigment-protein complexes under light-stress conditions. In this context it is of interest that ELIPs are very similar to proteins involved in carotenoid biogenesis (Lers et al., 1991) and that ELIPs have been shown to associate with light-harvesting complexes (Levy et al., 1992). As proposed by Levy et al. (1993) ELIPs could bind zeaxanthin to form photoprotective complexes within the light-harvesting antennas, which could quench surplus excitation energy (Horton et al., 1991).

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