In photosynthetic organisms chlorophyll and heme biosynthesis is tightly regulated at various levels in response to environmental adaptation and plant development. The formation of 5-aminolevulinic acid (ALA) is the key regulatory step and provides adequate amounts of the common precursor molecule for the Mg and Fe branches of tetrapyrrole biosynthesis. Pathway control prevents accumulation of metabolic intermediates and avoids photo-oxidative damage. In angiosperms reduction of protochlorophyllide (Pchlide) to chlorophyllide is catalyzed by the light-dependent NADPH:Pchlide oxidoreductase (POR). Although a correlation between down-regulated ALA synthesis and accumulation of Pchlide in the dark was proposed a long time ago, the time-resolved mutual dependency has never been analyzed. Taking advantage of the high metabolic activity of young barley (Hordeum vulgare L.) seedlings, in planta ALA synthesis could be determined with high time-resolution. ALA formation declined immediately after transition from light to dark and correlated with an immediate accumulation of POR-bound Pchlide within the first 60 min in darkness. The flu homologous barley mutant tigrina d12 uncouples ALA synthesis from dark-suppression and continued to form ALA in darkness without a significant change in synthesis rate in this time interval. Similarly, inhibition of protoporphyrinogen IX oxidase by acifluorfen resulted in a delayed accumulation of Pchlide during the entire dark period and a weak repression of ALA synthesis in darkness. Moreover, it is demonstrated that dark repression of ALA formation relies rather on rapid post-translational regulation in response to accumulating Pchlide than on changes in nuclear gene expression.

Keywords: Acifluorfen ● 5-Aminolevulinic acid ● Chlorophyll ● Dark repression ● FLU ● Heme ● Protochlorophyllide ● tigrina d12.

Abbreviations: ALA, 5-aminolevulinic acid; GluTR, glutamyl t-RNA reductase; GSAT, glutamate 1-semialdehyde aminotransferase; Pchlide, protochlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase; PPOX, protoporphyrinogen IX oxidase; Proto, protoporphyrin IX; Protogen, protoporphyrinogen IX; Proto(gen), accumulated protoporphyrinogen IX and protoporphyrin IX; PSII, photosystem II; TBARS, thiobarbituric acid reactive substances.

Introduction

In higher plants the formation of 5-aminolevulinic acid (ALA) is the first committed step in tetrapyrrole biosynthesis leading to the end products chlorophyll, heme, siroheme and phytöchromobilin (Beale 1999, Papenbrock and Grimm 2001, Tanaka and Tanaka 2007). The synthesis of tetrapyrroles is adjusted in response to changes in environmental conditions (e.g. light, temperature) and development. The regulation of cellular tetrapyrrole accumulation is inextricably linked to the formation of ALA, which is considered to be a rate-limiting step controlling influx into the entire pathway. ALA is formed in a three-step reaction including the ligation of glutamate to tRNA<sub>Glu</sub> catalyzed by glutamyl-tRNA synthetase, the reduction of glutamate to glutamate-1-semialdehyde by glutamyl-tRNA reductase (GluTR) and a final transamination step mediated by glutamate-1-semialdehyde aminotransferase (GSAT; Kannangara et al. 1988).

Light-dependent induction of ALA synthesis during de-etiolation and growth under dark/light conditions is well studied (Ilag et al. 1994, McCormack and Terry 2002). Within the metabolic pathway of tetrapyrrole biosynthesis ALA production is controlled by several feedback loops regulating gene expression or post-translational modifications. Signals are proposed to originate from heme and the Mg branch of the pathway. It is presently suggested that GluTR is the main target of regulatory mechanisms modulating ALA formation. The expression of HEMA1 encoding GluTR is regulated by
a wide range of stimuli (e.g. cytokinin, the circadian clock, plastid-derived signals and light), whereas GSA encoding GSAT responds only weakly (Cornah et al. 2003, Gough et al. 2003, Eckhardt et al. 2004, Tanaka and Tanaka 2007).

In vitro experiments the activity of barley GluTR was inhibited by heme (Pontoppidan and Kannangara 1994), which exerted the inhibiting action through the first 30N-terminal amino acids of the enzyme (Vothknecht et al. 1998). In *Chlamydomonas* an additional factor was required to mediate inhibition of GluTR by heme (Srivastava et al. 2005). Additionally, enzyme activities of the Mg branch feedback-control ALA synthesis. Tobacco mutants affected in the synthesis of CHLII or CHLH subunits of Mg chelatase not only showed decreased Mg chelatase activity, but also reduced ALA synthesizing activity. This metabolic feedback control acts at the transcriptional level (Papenbrock et al. 2000a, Papenbrock et al. 2000b) and prevents accumulation of tetrapyrrole intermediates generating reactive oxygen species upon excitation by light (Vavilin and Vermaas 2002).

ALA synthesis is drastically reduced in darkness. Previous observations revealed accumulation of the chlorophyll precursor protochlorophyllide (Pchlide) in etiolated seedlings of angiosperms, since Pchlide can be converted to chlorophyllide by the light-dependent NADPH:Pchlide oxidoreductase (POR) only (Griffiths et al. 1996). Several experiments with etiolated seedlings and green plants unveiled a strong inverse correlation between Pchlide accumulation and ALA synthesis in darkness (Fluur et al. 1975, Gough 1978, Ford and Kasemir 1980, Huang et al. 1984, Stobart and Ameen-Bukhari 1986). Thus, it was assumed that a high Pchlide level induces down-regulation of chlorophyll biosynthesis at the level of ALA synthesis (Beale 2006, Tanaka and Tanaka 2007).

The *Arabidopsis flu* mutant with a deficient negative regulator for ALA synthesis fails to down-regulate ALA synthesis in darkness and accumulates massive amounts of Pchlide (Meskauskiene et al. 2001). It was shown that FLU directly interacts with the *Arabidopsis* GluTR encoded by *HEMA1*, but not with the *HEMA2* product. Furthermore, the FLU-dependent inactivation of GluTR works independently of the above-mentioned GluTR inhibition by heme (Gaslings et al. 2004). Both the *Arabidopsis flu* mutant and the homologous barley *tigrina d*12 mutant (Lee et al. 2003) show a necrotic phenotype during photoperiodic growth emphasizing the importance of rapid and efficient down-regulation of ALA synthesis in darkness.

To date, very little is known about the nature of the rapid control resulting in repression of ALA formation. Conclusive evidence for the role of Pchlide in the dark repression of ALA synthesis is still missing. The present study shows in a time course analysis rapid changes in the ALA synthesis rate and Pchlide accumulation after light–dark transition in green barley leaves and provides evidence for an almost instantaneous repression of ALA synthesis in darkness in correlation with an accumulation of POR-bound Pchlide.

**Results**

**ALA synthesis in green barley leaves is immediately repressed in darkness**

Incubation of green barley leaves with levulinic acid, a competitive inhibitor of ALA dehydratase, facilitated time-resolved determination of ALA accumulation in both light and darkness. Several pre-experiments were performed to optimize our analysis of the in vivo metabolic activities. Using increasing amounts of levulinic acid we found that application of 40 mM levulinic acid was sufficient for rapid, reproducible accumulation of ALA within the experimental time period which correlates with the ALA-synthesis capacity (Supplementary Fig. S1a). Despite the detectable amounts of accumulated ALA due to inhibition by levulinic acid we found that Pchlide accumulation after 2 h of dark incubation was almost similar in leaves incubated with and without levulinic acid. These findings are explained by incomplete inhibition of ALA dehydratase (Supplementary Fig. S1b) allowing simultaneous analysis of ALA and Pchlide accumulation in the barley leaves.

Leaves exposed to light intensity of 100 μmol photons m−2 s−1 accumulated ALA at a constant rate of 260 pmol ALA mg FW−1 h−1 (Fig. 1a). After transfer to darkness ALA accumulation decreased instantaneously and continued at a very low rate (30 pmol ALA mg FW−1 h−1). When leaves were retransferred to light after 90 min dark incubation, enzyme activity was restored rapidly and ALA synthesis proceeded at the rate observed before dark incubation. Northern (Fig. 1b) and Western (Fig. 1c) blot analyses revealed no differences in expression of analyzed enzymatic steps of tetrapyrrole biosynthesis between light-exposed and dark-incubated samples with the exception of *HEMA1* RNA and POR protein. The POR level was elevated in darkness and was diminished during long exposure to light following diurnal oscillation. In green tissue the anti-POR antibody mainly recognizes PORB (Holtorf and Apel 1996). Unfortunately, the GluTR antibody available in the laboratory did not recognize the homologous barley protein, so we cannot prove influence of reduced *HEMA1* transcript level on GluTR amounts. However, a rapid increase in ALA synthesis after re-illumination reflects constant or elevated GluTR content.

**Pchlide accumulation in green barley leaves is stopped within one hour of dark incubation**

In darkness Pchlide is supposed to influence ALA formation by feedback inhibition (Reinbothe and Reinbothe 1996). Time-resolved Pchlide accumulation was monitored after light–dark transition of green barley leaves (Fig. 2). Within the first 30 min of dark incubation leaves accumulated up to 6-fold more Pchlide compared with Pchlide levels during illumination. Prolonged dark incubation (e.g. up to a 20 h period) did not significantly increase levels of accumulated Pchlide compared with short-term dark incubation during the experiments. When green leaves were re-exposed to light their Pchlide pool...
decreased and reached the steady-state level of illuminated leaves within minutes (Fig. 2).

These data correlate very well with the observation that ALA synthesis is rapidly down-regulated in darkness to avoid massive flux into the tetrapyrrole biosynthesis pathway. To address the question of whether Pchlide accumulates only in the photo-convertible POR-bound or also in the free non-photo-convertible form within the first 2 h of darkness, we performed 77K fluorescence analyses of crude barley leaf extracts followed by Gaussian deconvolution of fluorescence data to detect potential hidden maxima. Non-photo- and photo-convertible Pchlide (Ex 433 nm) show a fluorescence emission maximum at 635 nm and 650 nm, respectively (Böddi et al. 1992, Franck and Strzalka 1992). During dark incubation only an emission maximum at 650 nm was detectable, indicating the photo-convertible POR-bound form of Pchlide.
After 20 h dark incubation of young green barley leaves the fluorescence emission maximum at 635 nm can be assigned to free Pchlide (Fig. 3). The barley tigrina d12 mutant accumulated both the non-photo- and the photo-convertible Pchlide (Fig. 3).

**ALA synthesis is not suppressed in darkness when Pchlide levels do not increase**

As ALA synthesis in green barley leaves immediately declined after dark transition and Pchlide rapidly accumulated, it was important to perform additional experiments to confirm a Pchlide accumulation-dependent short-term feedback inactivation of ALA formation in darkness. Therefore, Pchlide accumulation in darkness was minimized by inhibiting the tetrapyrrole biosynthesis pathway upstream of Pchlide formation. The photosensitizing herbicide acifluorfen inhibits protoporphyrinogen IX oxidase (PPOX) resulting in a massive accumulation of protoporphyrinogen IX (Protogen). Fig. 4a displays the strongly reduced Pchlide formation in acifluorfen-treated and dark-incubated leaves compared with untreated leaves. It is worth mentioning that acifluorfen-treated leaves synthesized slightly more ALA than control leaves in light (Fig. 4b).

However, it is more important that ALA synthesis of acifluorfen-treated leaves was not reduced in darkness, while ALA synthesis of untreated leaves showed the expected dark repression (Fig. 4b). This indicates that ALA formation in darkness depends on the level of Pchlide in plastids and ALA synthesis can be uncoupled from dark-repression by acifluorfen-mediated prevention of Pchlide accumulation.

Heme is proposed to act as feedback inhibitor of ALA synthesis (Cornah et al. 2003, Goslings et al. 2004). To exclude possible heme effects, the heme content was determined in acifluorfen-treated and control leaves. The level of non-covalently bound heme was not reduced in acifluorfen-treated barley leaves, during neither light nor dark exposure (Table 1).

The acifluorfen treatment caused the expected massive accumulation of Protogen, which is rapidly oxidized to Proto (Witkowski and Halling 1988, Witkowski and Halling 1989). HPLC analysis revealed similar contents of Proto(gen) in dark and light-incubated acifluorfen-treated barley leaves, because ALA synthesis was not inactivated in darkness and resembled that of light-incubated leaf samples (Fig. 4c).

Eventually, treatment with acifluorfen leads to photodamage of plants (Lermontova and Grimm 2006). To exclude any adverse effects of acifluorfen on plastid integrity and physiology within the time frame of ALA synthesis analysis, photosynthetic parameters and membrane lipid peroxidation were determined. Tables 2, 3 point out that acifluorfen treatment of barley plants did not perturb photosynthetic processes within the first 6 h of illumination. The pigment content did not differ significantly between treated and untreated leaves (Table 2). Maximum quantum yield of PSII photochemistry, actual quantum yield of photochemical energy conversion in PSII and non-photochemical quenching of variable chlorophyll fluorescence in acifluorfen-treated leaves resembled control.

**Fig. 3** 77K fluorescence spectra of intact barley wild-type Lomerit (WT) leaves at different incubation times in light and darkness. Within 1 h of darkness only the POR-bound photoactive form of Pchlide accumulated having a fluorescence emission maximum at 650 nm (excitation at 433 nm). After 20 h of darkness initial accumulation of free non-photo-convertible Pchlide (emission at 635 nm) was observed. The fluorescence spectrum of tigrina d12 seedlings was obtained after 20 h of darkness. Spectra were normalized to chlorophyll fluorescence emission maximum at 680 nm. Using the Gaussian deconvolution method emission bands in the complex spectra were assessed.
leaves (Table 2). The content of thiobarbituric acid reactive substances (TBARS) did not differ significantly between control and acifluorfen-treated plants within the first 2 h of illumination indicating integrity of plastid membranes (Table 3). But continued photoperiodic growth for 2 days caused substantial phototoxic effects of acifluorfen as indicated by elevated amounts of TBARS in barley leaves compared with control values and leaf necrosis, which are explained by massive accumulation of Proto(gen) in herbicide-treated leaves (Table 3, Supplementary Fig. S2).

Table 1 Content of non-covalently bound heme in barley leaves

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Non-covalently bound heme (pmol mg FW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min light + 60 min light</td>
<td>Control</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Acifluorfen</td>
<td>7.6 ± 1.0</td>
</tr>
<tr>
<td>30 min light + 60 min dark</td>
<td>Control</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Acifluorfen</td>
<td>9.0 ± 1.4</td>
</tr>
</tbody>
</table>

Leaf discs of acifluorfen-treated and control barley plants were incubated in levulinic acid-containing buffer and exposed to light, or 30 min light followed by 60 min darkness. The amount of non-covalently bound heme was determined. Data are given as means ± SD.

Table 2 Photosynthetic parameters and pigment contents of barley leaves treated with acifluorfen for 20 h in darkness and subsequently transferred to light

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F_{v}/F_{m})</td>
<td>99.7 ± 1.0</td>
</tr>
<tr>
<td>(\Phi_{PSII})</td>
<td>102.5 ± 10.0</td>
</tr>
<tr>
<td>(qN)</td>
<td>104.2 ± 19.0</td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td>90.7 ± 7.3</td>
</tr>
<tr>
<td>Carotenoid content</td>
<td>90.4 ± 6.7</td>
</tr>
</tbody>
</table>

Acifluorfen-treated plants did not show any reduction in photosynthetic capacity compared with untreated controls within the first 6 h of light treatment. The pigment contents of untreated barley leaves were 287 ± 33 ng mg FW⁻¹ total chlorophyll and 125 ± 15 ng mg FW⁻¹ carotenoids. The chlorophyll \(a/b\) ratio was 4.2.

\(a\) Maximum quantum yield of PSII photochemistry.

\(b\) Actual quantum yield of photochemical energy conversion in PSII.

\(c\) Non-photochemical quenching of chlorophyll fluorescence.

Table 3 Membrane integrity of barley plants treated with acifluorfen for 20 h in darkness and subsequently transferred to light

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>TBARS (µmol mg FW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h light</td>
<td>Control</td>
<td>9.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Acifluorfen</td>
<td>10.1 ± 3.2</td>
</tr>
<tr>
<td>2 d light*</td>
<td>Control</td>
<td>11.4 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Acifluorfen</td>
<td>18.1 ± 1.5</td>
</tr>
</tbody>
</table>

Accumulation of TBARS corresponds to membrane integrity. Acifluorfen-treated leaves did not show any membrane disintegration after 2 h of light exposure, while membrane damage was indicated after 2 d of light/dark incubation.

\(a\) Photoperiodic growth.
All other attempts to inhibit Pchlide accumulation during dark incubation of barley leaves and to reproduce the results obtained in the presence of acifluorfen by independent experimental approaches were unsuccessful. Inhibitors, such as the iron-chelators dipyrød (Duggan and Gassman 1974) and thujaplicin (Oster et al. 1996) were applied similarly to acifluorfen, but prevented Pchlide accumulation in darkness less efficiently (data not shown). This may be attributed to limitations of inhibitor uptake through the leaf surface.

**Non-repressed ALA synthesis of the tigrina d12 mutant in darkness resembles response to acifluorfen treatment**

While dark-grown wild-type angiosperms immediately down-regulate ALA biosynthesis, the Arabidopsis flu and the homologous barley tigrina d12 mutants accumulate excessive amounts of Pchlide as a consequence of elevated ALA formation during the dark period (Hansson et al. 1997, Meskauskiene et al. 2001, Lee et al. 2003).

To substantiate the regulatory relationship between immediate dark-repression of ALA synthesis and rapid Pchlide accumulation, we included examinations of the kinetics of ALA synthesis and Pchlide accumulation of the tigrina d12 mutant and its wild-type variety Bonus in darkness. Bonus wild-type leaves followed the same pattern of light-stimulated and dark-repressed ALA synthesis as the wild-type variety Lomerit, but show a lower ALA synthesis rate in light compared with Bonus wild type (filled squares) rapidly repressed ALA synthesis while the mutant (filled circles) continued without decreasing enzyme activity. Data are given as means ± SD.

**Diminished ALA synthesis in low light also correlates with Pchlide accumulation**

ALA synthesis rate can be modulated over a range of increasing light intensities. It remains open whether ALA synthesis is attenuated at very low light intensities by the same regulatory mechanism as in darkness. ALA synthesis in green leaves of barley wild-type Lomerit was determined at light intensities <30 µmol photons m⁻² s⁻¹ to examine enzymatic activities at the transition between dark-repression and light-stimulation of ALA formation. Below 10 µmol photons m⁻² s⁻¹ ALA synthesis was reduced. In parallel the Pchlide steady-state level increased with decreasing light quantities (Fig. 6a). It is suggested that this elevated Pchlide level contributed to the diminished ALA synthesis rate at very low light intensities.

ALA synthesis and Pchlide levels at different light intensities were also determined in Bonus wild-type and tigrina d12 seedlings (Fig. 6b,c). The light-intensity dependency of ALA synthesis differed between tigrina d12 seedlings and its wild type. Consistent with the results of tigrina d12 ALA synthesis in darkness (Fig. 5), ALA synthesis was not reduced at low light intensity (3 µmol photons m⁻² s⁻¹), although the Pchlide levels (3 pmol mg FW⁻¹) were already elevated compared with that of wild type (0.55 pmol mg FW⁻¹).

**Discussion**

**Rapid post-translational control represses dark ALA synthesis in green leaves**

Light-dependent Pchlide reduction in angiosperms enables synthesis of new chlorophyll molecules exclusively upon light exposure. Pchlide is the only tetrapyrrole intermediate accumulating in darkness. It was previously demonstrated that Pchlide accumulation is inversely correlated with down-regulation of ALA synthesis in dark-grown green leaves and etiolated seedlings and therefore a regulatory role for Pchlide was proposed (Nadler and Granick 1970, Castelfranco et al. 1974, Fluhr et al. 1975, Gough 1978, Ford and Kasemir 1980, Stobart and Ameen-Bukhari 1984, Stobart and Ameen-Bukhari 1986, Huang and Castelfranco 1989). This regulatory mechanism is reasonable as down-regulated ALA synthesis in darkness prevents excessive accumulation of Pchlide. Its photoreactivity is harmful for light-exposed plants.

However, due to experimental limitations a time course analysis of the interdependence between Pchlide accumulation and activities of ALA synthesis has ultimately never been provided. Moreover, the proposed feedback control from Pchlide and POR could not be substantiated by protein–protein
isolated chloroplasts. A decline in ALA synthesis was demonstrated after the transfer of the samples to darkness. But the elucidation of rapid turn-off mechanisms of ALA synthesis was hindered by technical limitations. ALA synthesis was determined in leaves after levulinic acid incubation for several hours (Fluur et al. 1975, Huang and Castelfranco 1989, Beator and Kloppstech 1993, Kruse et al. 1997, Papenbrock et al. 1999, Goslings et al. 2004). Taking advantage of both high metabolic activities of green primary barley leaves and improved methodology ALA synthesis could be analyzed within 30 min.

In our experimental setup the synthesis of ALA was constant during light exposure and stopped almost instantaneously when leaf samples were transferred to darkness (Fig. 1). During the subsequent dark period a slow but continuing synthesis of additional ALA molecules was detected (Figs. 1, 4b). Observed differences at the level of transcripts and proteins involved in ALA formation may not explain the rapid dark-repression of ALA synthesis; an effective and rapid mechanism most likely acts at the post-translational level. In analogy, ALA formation recovers almost immediately and completely after dark to light transition (Fig. 1).

Under these assay conditions Pchlide accumulation in dark-incubated barley leaf samples reached a maximum within 30–60 min that did not further increase during prolonged dark periods. The underlying mechanism repressing ALA synthesis in darkness avoids Pchlide levels exceeding the binding capacity of POR and efficiently prevents accumulation of photosensitizing free Pchlide (Fig. 3). However, the molecular mechanisms of the light–dark switch of ALA formation are still not entirely elucidated.

**Inhibition of Pchlide accumulation hinders repression of ALA synthesis**

Our results corroborate the hypothesis of a negative feedback regulation of ALA formation in green tissues originating from the Mg branch of the tetrapyrrole biosynthesis pathway. When Pchlide accumulation was prevented, inactivation of ALA synthesis did not occur. This was achieved by inhibition of PPOX with acifluorfen that effectively stopped the metabolic flow in the tetrapyrrole biosynthetic pathway (Fig. 4; Witkowski and Halling 1988) without affecting the integrity of plastid physiology within the experimental time period (Tables 2, 3). However, Pchlide accumulation was inhibited (Fig. 4a) and, in turn, ALA formation was not repressed indicating a deficit in the repression signal in dark-incubated acifluorfen-treated leaves. This deregulation of ALA synthesis is rather attributed to deficient Pchlide accumulation than to increased levels of Proto(gen). Protogen and Proto are intermediates that do not accumulate in significant amounts, when green leaves are transferred from light to darkness. Moreover, transgenic tobacco plants with reduced amounts of the PPOX do not show deregulation of ALA formation in darkness (Lermontova and Grimm 2006).

Becerril et al. (1992) reported that acifluorfen-treated duckweed accumulated Proto(gen) in darkness over a period of 5 h.

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**Fig. 6** ALA (filled squares) and Pchlide (filled circles) accumulation in barley leaf discs of (a) wild-type Lomerit, (b) wild-type Bonus and (c) tigrina d12 incubated for 2 h in a buffer containing 40 mM levulinic acid at different light intensities (white light). In wild type but not in tigrina d12 ALA synthesis rate was decreased at light intensities <10 µmol photons m^{-2}s^{-1} down to a very low level in darkness. Data are given as means ± SD.
before ALA synthesis was stopped and the metabolite flow was negatively affected. It was suggested that acifluorfen treatment did not completely block PPOX leading to delayed Pchlide accumulation and Pchlide-mediated feedback inhibition of ALA synthesis. However, Pchlide accumulation is mandatory for efficient post-translational inactivation of ALA synthesis in darkness.

It was previously proposed that elevated levels of free heme control ALA synthesis in darkness (Masuda et al. 1990, Cornah et al. 2003, Goslings et al. 2004, Beale 2006) and GluTR activity was directly inhibited either by heme (Ponotoppidan and Kannangara 1994) or by an unknown plastid-localized factor (Srivastava et al. 2005). In our experiments the contents of non-covalently bound heme in light-exposed barley leaves did not differ from dark-incubated samples. Furthermore acifluorfen treatment did not result in significant reduction in the free heme content (Table 1). Since we determined the heme content of green tissue but not of isolated plastids we cannot fully exclude changes in the so-called free heme pool within plastids. As a light-dependent enzymatic step involved in heme metabolism has not been reported, yet, it is plausible that free heme accumulates rapidly after light to dark transition and contributes to the observed rapid dark-repression of ALA synthesis. It is likely that the remaining ALA synthesis in dark-incubated green leaves is directed into the Fe branch of tetrapyrrole biosynthesis, because maximum Pchlide accumulation was observed after 1h of dark incubation. It is not excluded that heme-dependent inhibition of ALA synthesis or GluTR activity reported previously, plays a role in an efficient long-term tuning of ALA synthesis in planta. It was shown that in light–dark grown tobacco plants heme accumulated and Fe chelatase activity increased in the dark periods (Papenbrock et al. 1999).

**POR and FLU are involved in rapid repression of ALA synthesis**

ALA synthesis was shown to be repressed at low light intensities with simultaneous accumulation of Pchlide (Fig. 6). Keeping in mind that ALA synthesis is almost immediately repressed in darkness and Pchlide accumulation occurs rapidly, it is conclusive that elevated Pchlide contents are almost instantaneously communicated to repress ALA synthesis by a post-translational mechanism. The regulatory mechanism of Pchlide-mediated dark repression of ALA synthesis involves possibly the ternary complex of NADPH:Pchlide POR (Oliver and Griffiths 1982). It is suggested that accumulating Pchlide bound to POR is responsible for the slow-down of ALA synthesis under low light conditions or in darkness (Fig. 6). Stobart and Ameen-Bukhari (1986) used ALA feeding experiments and titration with different amounts of ALA to investigate the light-induced ALA synthesis in etiolated barley seedlings. Both authors emphasized that Pchlide bound to POR affects light-stimulated ALA synthesis and free POR is mandatory to synthesize ALA. We proposed that due to its low content relative to etiolated tissue, POR is rapidly saturated with Pchlide in dark-incubated green tissue and this correlates with the fast post-translational inhibition of ALA formation.

The negative regulator FLU is probably a substantial part of the above-mentioned feedback control. FLU deficiency in the barley mutant *tigrina d12* prevents repression of ALA synthesis under low light intensities and in darkness and causes higher ALA synthesis during illumination (Figs. 5, 6). Further details of the feedback regulation sensing Pchlide accumulation in dark-incubated and low-light-exposed green leaves (Fig. 6) need to be elucidated. ALA formation is likely inhibited at the post-translational level. Future experiments are required to demonstrate that FLU also functions in response to light intensity. The current knowledge comprises Pchlide accumulation in darkness as the starting point of the feedback mechanism controlling ALA synthesis in green leaves. It is suggested that under low light intensities ALA synthesis experiences a similar Pchlide-induced repression. It is very likely that the FLU protein represents a signaling component that interacts directly with GluTR. The potential of FLU to inhibit GluTR activity in darkness and to modulate ALA synthesis at different light intensities in green tissues remains a subject of further investigation.

**Materials and Methods**

**Plant growth conditions**

Barley seedlings [*Hordeum vulgare* L. var. Lomerit and Bonus (wild type and *tigrina d12*)] were grown on vermiculite for 6 days in a 14 h light/10 h dark rhythm or continuous light, respectively, in a growth chamber at 22–23°C with normal light intensity of 80–100 µmol photons m⁻² s⁻¹.

**Experimental design**

Equal-sized primary leaves were harvested 2 h after onset of light and cut 3–4 cm below the tip into pieces of 1 cm length. Unless otherwise stated, 100 mg leaf material (corresponding to six leaf segments) was used for each measurement. For herbicide treatment 5-day-old barley seedlings were sprayed four times with 200 µM acifluorfen [in 10 mM Tris–HCl, pH 8.0, 0.05% (v/v) Tween 80] during a 20 h dark incubation.

For analysis of the ALA synthesis rate, RNA and protein expression, and levels of Pchilde, porphyrins and heme, leaf material was incubated in 50 mM Tris–HCl, pH 7.2 and 40 mM levulinate for 30 min at 22–23°C. After preincubation the samples were transferred to darkness for a time period indicated in the figure legends or remained under the same light condition. Standard light intensity was 100 µmol photons m⁻² s⁻¹. Different light intensities were applied as indicated. Samples were taken at different time points during the incubation time, dried on paper towels, weighed and frozen in liquid nitrogen. Leaf samples of 20 h dark-incubated acifluorfen-treated or control plants were subjected to measurements of TBARS and photosynthetic capacity at different time points after beginning of illumination.
Determination of ALA

ALA content was measured using the method of Mauzerall and Granick (1956). Frozen samples were homogenized, resuspended in 20 mM potassium phosphate buffer pH 6.8 and centrifuged for 10 min at 16,000×g; 400 µl of the supernatant was mixed with 100 µl of ethyl acetoacetate and boiled for 10 min at 100°C. Samples were mixed with 500 µl of modified Ehrlich’s reagent [373 ml of acetic acid, 90 ml of 70% (v/v) perchloric acid, 1.55 g of HgCl₂, 9.10 g of 4-dimethylaminobenzaldehyde and 500 ml of H₂O] and centrifuged for 5 min at 16,000×g. Absorption was measured at 526, 553 and 720 nm and the ALA content of the samples was calculated using a standard curve generated by commercial ALA (Sigma-Aldrich Inc.).

Determination of the Pchlide content

Extraction of Pchlide followed the protocol of Koski and Smith (1948). Leaf samples were fixed with steam for 2 min, frozen, ground in liquid nitrogen and extracted three times in alkaline acetone (9:1, 100% acetone: 0.1 N NH₄OH). After centrifugation at 16,000×g for 10 min the supernatants were collected and chlorophyll was removed by a stepwise extraction with 1 vol, 1 vol and 0.3 vol of 100% n-hexane. The Pchlide content of the samples was quantified using HPLC according to Langmeier et al. (1993). Samples were injected and separated by a reverse phase column (Waters RP-18 ODS Hypersil 3 µM, followed by a 2 min isocratic run with solvent A. The flow rate and chlorophyll was removed by a stepwise extraction with 7-day-old etiolated barley leaves (Koski and Smith 1948) and quantified using the extinction coefficient εₚchlide (398 nm) of 144 mM⁻¹ cm⁻¹ (Weinstein and Beale 1983).

Determination of pigments and other porphyrins

Chlorophyll and carotenoids were extracted using 80% (v/v) acetone with 10 µM KOH and determined spectrophotometrically using the extinction coefficient εₕeme (378 nm) of 144 mM⁻¹ cm⁻¹ (Weinstein and Beale 1983).

RNA analysis

Plant total RNA was extracted following the TRIzol protocol (Bioline GmbH, Luckenwalde, Germany). Ten micrograms of each sample was separated electrophoretically and transferred to Hybond N+ membrane (GE Healthcare) using standard protocols (Sambrook and Russel 2001). Probes were amplified by PCR from a barley cDNA using gene-specific primers for HvHEMA1 (5′-GCCAGCCGGGGGGCGACTTCCGGCGGCAAG-3′, 5′-TGCCAATTTCTGACAACTTGTTGACT-3′, HvPORB (5′-GCCGCCACTTCTCTTCGCGTCG-3′, 5′-ACATGGAGAACATGGCCAGCGTCC-3′) and HvCAB (chlorophyll a/b binding protein 5′-TCTGTCCTTCCTCACTTCCGCGGGG-3′, 5′-ACATGGAGAACATGGCCAGCGTC-3′) and labelled with [α-32P]dCTP using random oligonucleotide priming (HexaLabel DNA labelling Kit; Fermentas GmbH, Germany). Prehybridization and hybridization were performed in Church buffer (Church and Gilbert 1984) at 70°C. Final washings were carried out to a stringency of 0.1×SSC and 0.1% (w/v) SDS at 65°C.

Protein analysis

Plant total protein was extracted by grinding frozen leaf material in a buffer containing 2% (w/v) SDS, 56 mM NaCl, 12% (w/v) sucrose, 56 mM DTT and 2 mM EDTA, pH 8.0, followed by heating at 70°C for 20 min and centrifuging at 16,000×g for 10 min. The protein concentration in the supernatant was measured using the bicinchoninic acid assay (BCA) method (Pierce).
measured using BCA protein assay reagent (Perbio Science) after trichloroacetic acid precipitation. Proteins were separated on 12% or 15% polyacrylamide gels, transferred to Hybond-C membranes (GE Healthcare) and probed with specific antibodies using standard protocols (Sambrook and Russel 2001).

**Determination of membrane lipid peroxidation**

Approximately 200 mg leaf material was homogenized in 0.25% (w/v) thiobarbituric acid in 10% (w/v) TCA and heated at 95°C in a glass tube in a water bath for 30 min to form malondialdehyde according to De Vos et al. (1989). The amount of TBARS was calculated from the difference in absorbance at 532 nm and 600 nm using an extinction coefficient ε(532 nm – 600 nm) of 155 mM⁻¹ cm⁻¹.

**Measuring the photosynthetic capacity**

Chlorophyll fluorescence parameters were measured using a PAM 2000 (Walz, Effeltrich, Germany). Conventional fluorescence nomenclature was used (van Kooten and Snel 1990, Rohácek 2002). Attached leaves were dark adapted for 30 min for determination of the maximum quantum yield of PSII photochemistry (calculated as ratio Fm/Fo = (Fm – Fo)/Fo). After determining the basic fluorescence (Fo) an 800 ms saturating pulse was applied to ascertain the maximum fluorescence (Fm). The actual quantum yield of photochemical energy conversion in PSII, was determined according to Genty et al. (1989) on growth light-adapted leaves. The non-photochemical quenching of variable chlorophyll fluorescence, qN, was calculated by the formula qN = 1 – (Fm’ – Fo’)/(Fm – Fo). Fm and Fo were determined as described above following the determination of Fm’ and Fo’ upon 15 min adaptation to actinic light at an intensity appropriate to growth light intensity.

**Miscellaneous**

All experiments were performed with three to six independent experiments. In order to test significant differences between calculated values equality of variances was tested by a F-test followed by Student’s t-test using a P-value of <0.05.

**Supplementary data**

Supplementary data are available at PCP online.

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