Expression of Enzymes Involved in Chlorophyll Catabolism in Arabidopsis Is Light Controlled[1][W]

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We found that the levels of mRNA of two enzymes involved in chlorophyll catabolism in Arabidopsis (*Arabidopsis thaliana*), products of two chlorophyllase genes, *AtCLH1* and *AtCLH2*, dramatically increase (by almost 100- and 10-fold, respectively) upon illumination with white light. The measurements of photosystem II quantum efficiency in 3-(3,4-dichlorophenyl)-1,1-dimethylurea-inhibited leaves show that their expression is not related to photosynthesis but mediated by photoreceptors. To identify the photoreceptors involved, we used various light treatments and Arabidopsis photoreceptor mutants (*cry1*, *cry2*, *cry1cry2*, *phot1*, *phot2*, *phot1phot2*, *phyA phyB*, *phyAphyB*). In wild-type Columbia, the amount of transcripts of both genes increase after white-light irradiation but their expression profile and the extent of regulation differ considerably. Blue and red light is active in the case of *AtCLH1*, whereas only blue light raises the *AtCLH2* mRNA level. The fundamental difference is the extent of up-regulation, higher by one order of magnitude in *AtCLH1*. Both blue and red light is active in the induction of *AtCLH1* expression in all mutants, pointing to a complex control network and redundancy between photoreceptors. The blue-specific up-regulation of the *AtCLH2* transcript is mediated by cryptochromes and modulated by phototropin1 and phytochromes. Individually darkened leaves were used to test the effects of senescence on the expression of *AtCLH1* and *AtCLH2*. The expression profile of *AtCLH1* remains similar to that found in nonsenescing leaves up to 5 d after darkening. In contrast, the light induction of *AtCLH2* mRNA declines during dark treatment. These results demonstrate that the expression of enzymes involved in chlorophyll catabolism is light controlled.

Light is the very essential factor that drives and regulates most activities of plants, in particular their growth and development. The majority of responses to light are either mediated by specialized photoreceptors containing light-sensing chromophores or are related to photosynthesis. Three major classes of plant photoreceptors have so far been identified—phytochromes, which sense light in the red and far-red region of the spectrum, blue-light-sensitive cryptochromes, and phototropins. The former two types of photoreceptors have been found in the nucleus, where they can interact directly with transcription factors (Guo et al., 1999; Wang et al., 2001; Kircher et al., 2002). The nuclear localization of phototropins still is unclear. In darkness they remain membrane bound but upon blue-light irradiation, phot1 moves rapidly to the cytoplasm in etiolated Arabidopsis (*Arabidopsis thaliana*) seedlings (Sakamoto and Briggs, 2002), while a frac-

tion of phot2 localizes to the Golgi apparatus in epidermal cells of seedlings, mesophyll protoplasts, and guard cells (Kong et al., 2006). Recently, phot2 has been reported in the nucleus of mesophyll cells (Jeong et al., 2010). However, this finding needs further confirmation. The subcellular localization of the photoreceptors is obviously related to their functioning. Phytochromes and cryptochromes are involved in the regulation of gene expression in Arabidopsis seedlings, where phototropins show only minor effects (Ma et al., 2001; Tepperman et al., 2001; Ohgishi et al., 2004). In contrast, the main activity of phototropins lies in the regulation of movement (Jarillo et al., 2001; Kinoshita et al., 2001; Sakai et al., 2001).

Apart from photoreceptors, light can trigger the expression of nuclear genes via photosynthesis-related signals, which include the redox state of plastids, the metabolic state of the cell, reactive oxygen species, or photosynthesis-delivered carbohydrates (for review, see Kleine et al., 2009; Pfannschmidt et al., 2009). Depending on plant species, the redox signals have been shown to act in retrograde signaling that involves two different sites, the plastoquinone pool or the PSI acceptor site (Kruk and Karpinski, 2006). The latter seems to be active in Arabidopsis (Baier et al., 2004).

Seasonal changes in vegetation and the senescence of plants involve a massive turnover of chlorophylls (Chls) in the biosphere, amounting to over 1 billion tons of these pigments biosynthesized and biodegraded per annum (Brown et al., 1991; Gossauer and Engel, 1996; Matile, 2000). For long, the spectacular phenomenon of the biological breakdown of Chla remained poorly

understood and was considered a biological enigma (Hendry et al., 1987). Only in the last decade or so, since the characterization of several intermediate catabolites of Chl accompanied by advances in molecular biology methods for identifying the enzymes involved, has the process appeared less enigmatic (Gossauer and Engel, 1996; Hörtensteiner, 1999; Kräutler and Matile, 1999; Matile, 2000; Oberhuber et al., 2003; Kräutler and Hörtensteiner, 2006; Kräutler, 2008). Now it is seen as a sequence of well-synchronized steps in the safe withdrawal of Chl molecules, which, once released from photosynthetic complexes, become photocytotoxic due to the photodynamic effect (Fiedor et al., 1993, 2001). The withdrawal of Chls also seems correlated with other processes of salvaging thylakoid proteins and lipids (Ischebeck et al., 2006).

Chlorophyllase (Chlase), the first plant enzyme discovered a century ago by Willstätter and Stoll, and already then suggested to be an integral part of the Chl biodegradation pathway (Willstätter and Stoll, 1913), is among the key enzymes involved in the process. The enzyme catalyzes, with a high degree of stereospecificity, the splitting of the Chl molecule into the carboxylic acid chlorophyllide (Chlide) and phytol (Fiedor et al., 1992, 1996). This step, after which polar Chlide may leave the lipid membrane environment, seems a prerequisite for the further biodegradation of Chl. Chlide becomes available to magnesium dechelatase and then, as pheophorbide, is quickly converted via other degradative enzymes to more polar products that end up in vacuoles (Gossauer and Engel, 1996; Hörtensteiner, 1999; Kräutler and Matile, 1999; Matile, 2000; Oberhuber et al., 2003; Kräutler and Hörtensteiner, 2006; Kräutler, 2008). Recently, it has been proposed that during the leaf senescence in Arabidopsis the step of magnesium removal from Chl precedes hydrolytic degradation, and the latter is catalyzed not by Chlase but by another enzyme called pheophytinase (Schelbert et al., 2009). Despite the essential role of Chls, little information is available as to how their catabolism is regulated and what triggers and controls the activity of the particular enzymes involved. Chlase seems to be regulated at the level of gene induction and expression; ethylene is the best known among the inducers of de novo synthesis of the enzyme, both at the level of mRNA and protein (Trebitsh et al., 1993; Jacob-Wilk et al., 1999; Azoulay-Shemer et al., 2008). However, there are many plant species in which Chlase is constitutive and substantial levels of the enzyme can be found during the entire vegetation season (Hendry et al., 1987; Brown et al., 1991; Fiedor et al., 1992, 2003). In Arabidopsis, two genes, *AtCLH1* and *AtCLH2*, have been found to encode Chlases. The level of *AtCLH1* mRNA is enhanced by jasmonic acid (Tsuchiya et al., 1999), wounding, or pathogen attack (Kariola et al., 2005), and decreases during dark treatment (Lin and Wu, 2004). The amount of *AtCLH2* mRNA remains unaffected in these conditions but its expression appears correlated with ozone levels (Mahalingam et al., 2006).

While light is known to up-regulate photosynthesis-related genes mainly via phytochrome (Tepperman et al., 2001), no information is available on its influence on the genes responsible for Chl catabolism. Senescence in the canopy is accelerated by high far-red/red-light ratio (Rousseaux et al., 2000). The most visible effect of senescence is Chl degradation. For instance, illumination of tobacco (*Nicotiana tabacum*) leaves with far red causes a drop in Chl content, which does not occur in *Nicotiana* plants overexpressing oat (*Avena sativa*) *phyA* (Rousseaux et al., 1997). We have observed that the levels of mRNA of two enzymes involved in Chl catabolism in Arabidopsis, products of the *AtCLH1* and *AtCLH2* genes, dramatically increase (by almost two and one order of magnitude, respectively) upon illumination with white light. This prompted us to investigate in detail the mechanism of the effects caused by light. To identify the photoreceptors involved in the expression of the *AtCLH1* and *AtCLH2* genes, the responses of the respective mRNA levels to monochromatic blue and red light were compared in a series of photoreceptor mutants of Arabidopsis. The relationship between the expression of the *CLH* genes and senescence was studied using the standard model of dark-induced leaf senescence.

RESULTS

Light Effects on the Level of *AtCLH1* and *AtCLH2* Transcripts

The relative levels of *AtCLH1* and *AtCLH2* mRNA were estimated in Arabidopsis wild-type Columbia leaves using the quantitative reverse transcription (qRT)-PCR technique. In the dark, the accumulation of *AtCLH1* mRNA was about 16-fold higher than that of *AtCLH2* (data not shown). As demonstrated in Figure 1, illumination with white light for 3 h (120 μmol m^{-2} s^{-1}) resulted in a strong increase in the transcript level for both genes: 100-fold for *AtCLH1* and 20-fold for *AtCLH2*. In the next step, to clarify the mechanism underlying the light control of the mRNA levels of *AtCLH1* and *AtCLH2*, the influence of blue and red illumination was examined. While both blue and red light were active in the case of *AtCLH1* (Fig. 2A, wild type), the mRNA level corresponding to *AtCLH2* was affected only by blue light (Fig. 2B, wild type). Again, a much higher up-regulation was observed for *AtCLH1*: 24-fold by red- and over 30-fold by blue-light illumination, while it was only 2-fold for *AtCLH2* in the blue-light-treated leaves. Similar results were obtained for Landsberg *erecta* plants, the background for phytochrome mutants (data not shown).

Light Regulation of the *AtCLH1* and *AtCLH2* Transcript Level in 3-(3,4-Dichlorophenyl)-1,1-Dimethylurea-Treated Leaves

To check whether photosynthesis is involved in light regulation of the *AtCLH1* and *AtCLH2* transcript
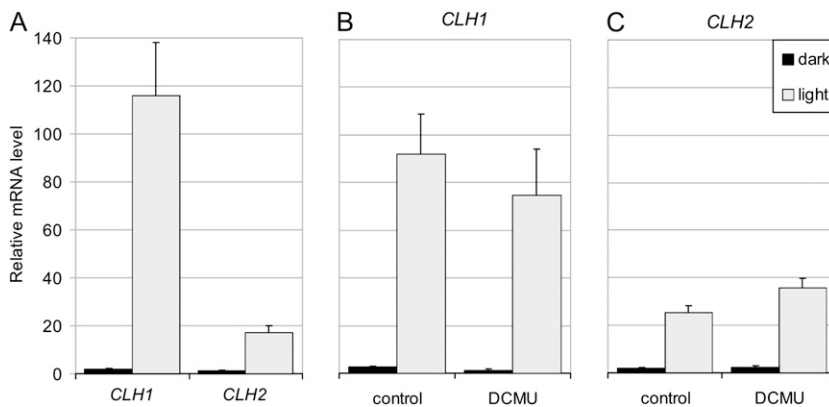
Figure 1. The effect of white light (3 h irradiation with 120 μmol m^{-2} s^{-1}) on the level of the *AtCLH1* and *AtCLH2* transcripts in Arabidopsis leaves (4- to 6-week-old plants). A shows relative mRNA levels of *AtCLH1* and *AtCLH2* in the dark-adapted (black bars) and in the irradiated leaves (gray bars). B and C are the expression of *AtCLH1* and *AtCLH2*, respectively; black bars: effects of 200 μM DCMU in 0.4% DMSO; gray bars: effects of 0.4% DMSO (control). Each bar represents an average of measurements for at least seven leaves from different plants. All qRT-PCR reactions were run in duplicate. Error bars indicate SE.

accumulation, respective levels of mRNA were determined in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated leaves. The extent of inhibition of photosynthesis was estimated on the basis of Chl fluorescence from the photosystems. Treatment with DCMU caused a 40% to 50% drop in the quantum efficiency of PSII (Table I). The white-light-induced expression of both *AtCLH1* and *AtCLH2* was not inhibited under these conditions (Fig. 1). The former one shows a distinct increase and the latter equals the control value when the respective changes in the dark levels are taken into account.

The *AtCLH1* and *AtCLH2* Expression in Photoreceptor Mutants

To identify the photoreceptors involved in the regulation of the *AtCLH1* and *AtCLH2* mRNA levels, single and double knockout mutant plants were used, including the phytochrome (*phyA*, *phyB*, *phyAphyB*), cryptochrome (*cry1*, *cry2*, *cry1cry2*), and phototropin (*phot1*, *phot2*, *phot1phot2*) deficient lines of Arabidopsis.

The general pattern of up-regulation of *AtCLH1* expression with blue light more effective than red light was preserved in the mutants (Fig. 2A), although they differ in the extent of transcript accumulation. The blue-light-induced up-regulation of the *AtCLH1* transcripts, in the range of 30-fold, typical of the wild-type plants, was conserved for the *phot2* and *phot1phot2* mutants. In all phototropin mutants also the red-light effect was comparable to the one observed in wild type. The steady-state levels of *AtCLH1* mRNA recorded after illumination with blue and red light were significantly lower in cryptochrome and phytochrome mutants, especially in the *cry2* mutant line. The lowest red-light-induced accumulation of *AtCLH1* transcripts was observed in the *cry2*, *phyA*, and double *phyAphyB* mutants. The phytochrome mutants had similar level of *AtCLH1* mRNA after blue-light treatment, but the dark levels in *phyB* and *phyAphyB* plants were significantly lower than in wild type (about one-fifth of that in wild type). Taking this into account, one may consider the blue-light-induced *AtCLH1* transcript accumulation as more effective in these plants than in wild type.

A different pattern of light regulation was obtained with *AtCLH2*. In the wild-type plants, the level of *AtCLH2* mRNA increased 2-fold after irradiation with blue but not with red light (Fig. 2B). In all photoreceptor mutants tested, the dark level was lower than in wild type, falling below 25% of the wild-type level in *cry1*, *cry2*, and all phytochrome mutants. Insignificant light effects were observed in the double mutant *cry1cry2*. Moreover, when compared to wild type, the *cry1* and *cry2* mutants showed lower amounts of transcripts both in darkness and after irradiations. Although the light induction was conserved in these mutants, their *AtCLH2* mRNA levels, even after irradiation, were below that measured for wild type in the dark. On the contrary, blue-light accumulation of this transcript was higher in the *phot1* and *phot1phot2*, but not in *phot2*. Upon blue light, the steady-state levels of *AtCLH2* mRNA appeared comparable in phytochrome mutants and wild type. However, blue light appears to cause significantly higher transcript accumulation than in wild type, when their low dark level is taken into account. Interestingly, red light caused an increase of the *AtCHL2* mRNA level in all phototropin mutants and in the mutants lacking phyB (*phyB* and *phyAphyB*). This red-light effect, much weaker than the blue one, was not seen in wild type.

It has to be stressed that, similar to wild type, all changes in *AtCLH2* transcript levels stimulated by light in the photoreceptor mutants were lower by at least one order of magnitude than those recorded for *AtCLH1*.

Expression of *AtCLH1* and *AtCLH2* in Senescing Leaves

To verify the correlation of *AtCLH1* and *AtCLH2* mRNA accumulation with senescence we used a model commonly applied in senescence studies (Weaver and Amasino, 2001). The levels of mRNA were analyzed in leaves darkened individually on a plant growing in the standard photoperiod. A darkened leaf was either illuminated for 3 h or kept in the dark before being collected. The level of *AtCLH1* mRNA in the nonirradiated leaves markedly decreased after the initial 24 h of dark treatment from 3 to below 1 (Fig. 3A). However, the *AtCLH2* transcript level was not down-regulated

Figure 2. Light regulation of *AtCLH1* and *AtCLH2* transcript levels in photoreceptor-deficient Arabidopsis plants. The relative level of *AtCLH1* (A) and *AtCLH2* (B) mRNA in dark-adapted leaves (black bars), and after 3 h irradiation with 40 μmol m^{-2} s^{-1} of blue (white bars) or red light (gray bars). The values obtained for wild-type plants are additionally marked with stripes. The photoreceptor mutants used are specified under the respective bars. Each bar represents an average of measurements for at least 10 leaves from different plants pooled together. All qRT-PCR reactions were run in triplicate. Error bars indicate SE.
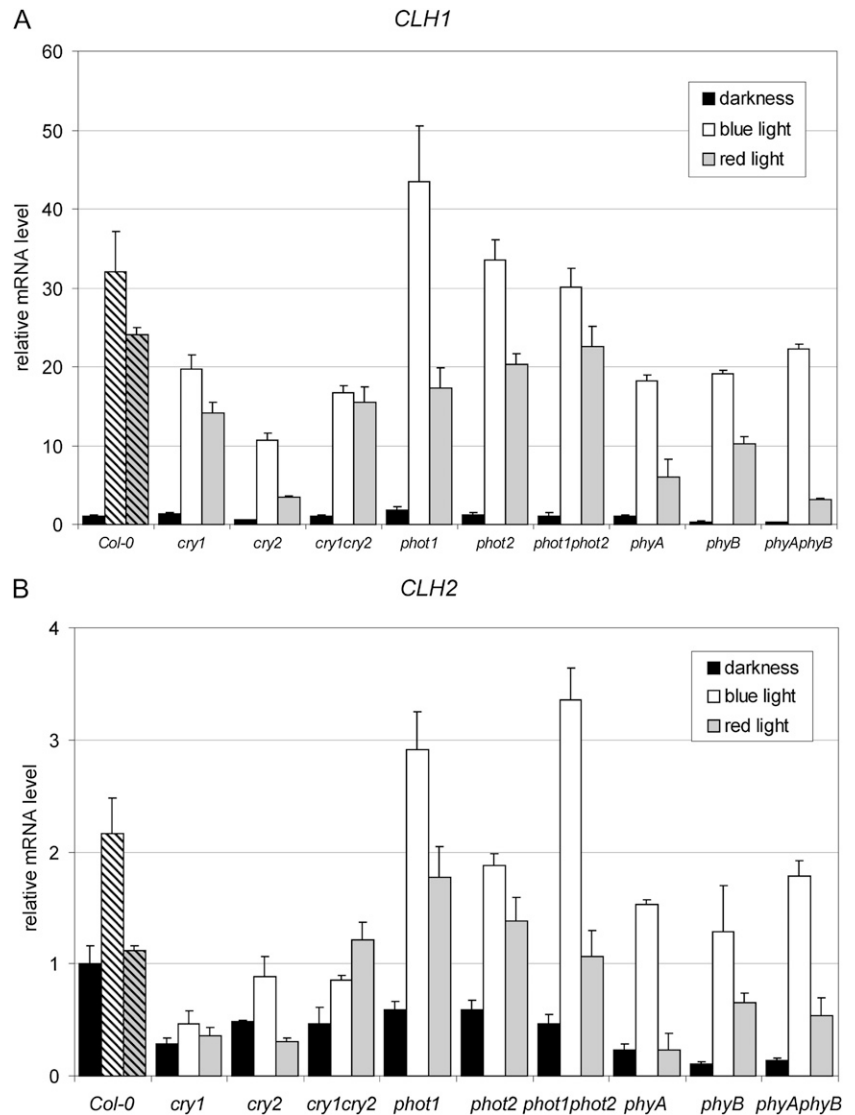
even upon prolonged darkening, and showed only some fluctuations over time, ranging between 1.5 and 3.5 (Fig. 3B). The sensitivity of the two genes to light behaved in an opposite way. Whereas the level of up-regulation of *AtCLH2* by light declined in time from 40- to 7-fold, *AtCLH1* light responsiveness during the entire period of dark-induced senescence was near 50-fold, still toward the end of the experiment being comparable to that of nonsenescing leaves.

DISCUSSION

One of the most important processes regulated by light, which involves specific photoreceptors, is the assembly of photosynthetic apparatus. The assembly of photosynthetic machinery during deetiolation, i.e. Chl biosynthesis and chloroplast development, is controlled by light. δ-Aminolevulinic acid is one of the first intermediate metabolites in Chl biosynthesis.

Genes encoding enzymes involved in its synthesis, i.e. Glu 1-semialdehyde 2,1-aminomutase (*GSA1*) and Glu-tRNA reductase (*HEMA*), are up-regulated by light (Ilag et al., 1994). In Arabidopsis seedlings, the expression of *HEMA1* is strongly enhanced by red, far-red, and blue light in a phytochrome-dependent manner (McCormac and Terry, 2002). Another key step in

Table I. *Quantum efficiency of PSII measured in Arabidopsis wild-type dark-adapted leaves*

After the measurements, the leaves were taken for testing light effects on the mRNA levels (results shown in Fig. 1, B and C, see the text for details). The SD values of the estimations are also indicated.

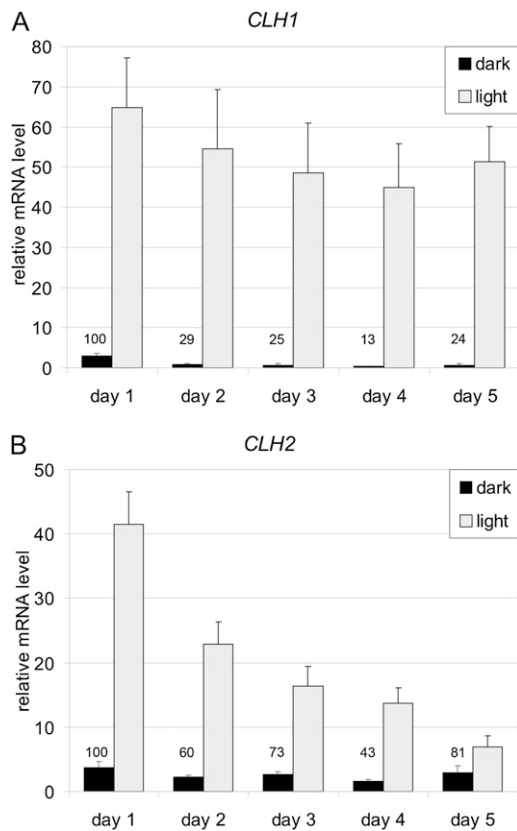| Leaf Treatment	PSII Quantum Efficiency	SD
No treatment	0.818	0.004
Incubation with 0.4% DMSO	0.803	0.011
Incubation with 200 μM DCMU in 0.4% DMSO	0.496	0.066

Figure 3. The expression of *AtCLH1* and *AtCLH2* during dark-induced leaf senescence. Individual leaves were wrapped in black paper and kept in darkness for the number of days as specified under the bars, while the plant was grown in standard photoperiod. The relative level of *AtCLH1* (A) and *AtCLH2* (B) in darkened leaves (black bars) and after 3 h of 120 μmol m^{-2} s^{-1} white-light irradiation (gray bars). The dark levels are indicated above the respective bars as percentage of the value for day 1. Each bar represents an average of measurements for at least six leaves coming from different plants. All qRT-PCR reactions were run in duplicate. Error bars indicate SE.

Chl biosynthesis is the reduction of protochlorophyllide to Chlide catalyzed by the light-dependent protochlorophyllide/protochlorophyllide oxidoreductase (POR). Both the activity and expression of this enzyme are controlled by light. Interestingly, after illumination the mRNA level of *PORA* decreases, that of *PORB* remains unchanged, and for *PORC* increases (Oosawa et al., 2000).

Our present results clearly demonstrate that the expression of the enzymes involved in Chl catabolism, *AtCLH1* and *AtCLH2*, is also strongly up-regulated by light in the visible region. Remarkably, the increase in the AtCLH1 mRNA level is over 15-times larger than that of *AtCLH2*. Such substantial differences in the light regulation of the two transcript levels have been revealed in all experiments. The roles of AtCLH1 and AtCLH2 in Chl catabolism are a matter of debate (Schenk et al., 2007) but, clearly, the up-regulation of Chl degradation would reduce phototoxic effects of Chls released from the photosystems.

The inhibition of photosynthesis by DCMU is a standard procedure for testing the influence of photosynthesis on transcriptome regulation (Pfannschmidt et al., 2009). The herbicide acts as a site-specific electron transport inhibitor, preventing a reduction of plastoquinone and, in turn, the redox state of plastoquinone pool affects the expression of nuclear genes. The fact that DCMU has no inhibitory effect on the accumulation of both *AtCLH1* and *AtCLH2* transcripts implies that the effects of white light are not related to photosynthesis. Therefore, a line of photoreceptor-deficient mutants was used to reveal the pattern of light regulation involved in *AtCLH1* and *AtCLH2* expression. The stronger up-regulation of both genes by white light than by red or blue light alone, points to synergistic effects of several photoreceptors acting in different regions of the spectrum.

The genes encoding AtCLH1 and AtCLH2 are controlled by photoreceptors in different ways. The expression of *AtCLH1* is enhanced by both red and blue light. The pattern revealed does not point to any single photoreceptor or group of photoreceptors dominating in the blue-light control of *AtCLH1* transcript enhancement. Blue light enhanced the expression of *AtCLH1* in all tested mutants ranging from 15 to over 80 times as compared to the respective dark level. Red-light enhancement ranged from 10 to 30 times with exception of *cry2* and *phyA* mutants. Hence, the light effect on *AtCLH1* appears to be mediated by an interplay of photoreceptor pigments with cryptochromes (mainly cry2) acting as positive modulators in blue light. PhyA and cry2 seem to be especially important in red-light regulation. Since cryptochromes perceive blue, but not red light, they cannot be involved directly in red-light signaling. Rather, some components of red-activated signaling pathway may be lacking in the *cry2* mutant. Although phyA is mainly responsible for far-red-induced responses, this photoreceptor has also been shown to control gene expression under continuous red light (Molas and Kiss, 2008). Only recently, phyA has been shown to be active under continuous red light with fluence rates higher than 50 μmol m^{-2} s^{-1}. Moreover, continuous red light of over 160 μmol m^{-2} s^{-1} not only did not cause degradation of phyA protein, but even stabilized phyA in its active form in Arabidopsis seedlings (Franklin et al., 2007).

In contrast, blue light clearly dominates in the up-regulation of *AtCLH2*. The lower levels of *AtCLH2* transcripts in both *cry* mutants and the weak light effect in the double mutant point to cryptochromes as the key photoreceptors redundantly involved in the blue-light control of *AtCLH2* expression, with a dominant role of cry1 photoreceptor. While the *phyA* mutants behave similarly to wild type in this, that the regulation occurs only via blue light in these plants, the red light is effective in *phyB*. The same tendency is reflected in the *phyAphyB* double mutant and in the phototropin mutants. These results point to an inhibitory role of phyB in *AtCLH2* accumulation in red light, with possible modulating activity of phototropins. To sum up, the

accumulation of *AtCLH2* is slightly promoted by red light via phytochromes other than phyB; this effect is counteracted by phyB coacting with phototropins.

A noteworthy effect is the blue-mediated enhancement of *AtCLH2* transcript accumulation in the *phot1* mutant, reflected also in *phot1phot2*, stronger than that observed in wild type. This result may be interpreted in terms of phot1 acting as a negative regulator of the cry-mediated elevation of the *AtCLH2* transcript upon blue-light treatment. Phototropins are generally recognized as photoreceptors eliciting plant organ, cell, and/or subcellular movements, acting on established ready-to-use protein systems rather than at the gene expression stage (Jiao et al., 2003; Ohgishi et al., 2004). However, in *Chlamydomonas*, phototropin has been reported to control enhanced expression of several photosynthesis-related genes in weak blue light (Im et al., 2006). In Arabidopsis, phot1 has been shown to be responsible for the blue-light-dependent destabilization of Lhcb and rbcL mRNAs (Folta and Kaufman, 2003). Thus, phot1 may play a role in the optimization of photosynthesis not only by controlling chloroplast distribution in the cell and stomatal aperture, but also by regulating the transcripts of the genes involved in photosynthesis. In view of the above observations, the negative regulation of cry1/cry2-mediated elevation of the *AtCLH2* transcript upon blue-light treatment might be caused by the destabilization of this transcript. This explanation, however, requires further verification. On the other hand, at the present, we are unable to interpret the red-light enhancement of *AtCLH2* expression in phototropin mutants. Interestingly, a similar increased sensitivity to red light has been previously reported in *phot2* plants (Krzeszowiec et al., 2007).

Many authors report on circadian regulation of light-induced genes (Millar and Kay, 1996; Harmer et al., 2000; for review, see Jiao et al., 2007). Moreover, phytochromes and cryptochromes, which appear to be involved in the regulation of Chlases mRNA accumulation have been shown to be also involved in the regulation of circadian rhythms. They also show a rhythmic transcription (McWatters and Devlin, 2011). Nevertheless, analysis of microarray data available at diurnal.cgrb.oregonstate.edu does not show regulation of either *AtCLH1* or *AtCLH2* in the circadian clock-dependent manner. The microarray data have been obtained using 7- to 9-d-old-seedlings, thus a possibility that circadian regulation of these two genes occurs in mature leaves cannot be ruled out. This, however, requires further investigations.

Light regulation of *AtCLH2*, but not of *AtCLH1*, diminishes with time in individually darkened leaves. This is yet another feature that points to differences in the light regulation of the transcript levels for both enzymes. As mentioned above, it has been suggested that pheophytinase, not Chlase, is involved in Chl degradation during Arabidopsis leaf senescence (Schenk et al., 2007; Schelbert et al., 2009). In both cases, the senescence was induced by darkening detached leaves. In our experiments, the levels of mRNA of both

AtCLH1 and *AtCLH2* were markedly increased by light. This implies that light might be an important factor that is required for Chl degradation during senescence. In consequence, the protocol commonly used (also in our experiments) to achieve senescence by darkening may be inadequate for studies assessing the role of Chlases in this process. Other models of senescence have to be tested to address the question whether enzymes encoded by *AtCLH1* and *AtCLH2* are functional during regular Chl turnover or during senescence.

CONCLUSION

Our experiments demonstrate in a straightforward manner that the expression of enzymes involved in Chl catabolism, the products of the *AtCLH1* and *AtCLH2* genes, is light controlled, both during regular Chl turnover and senescence. The light effect on *AtCLH1* is mediated by an interplay of photoreceptors, with cryptochromes and phyA being positive modulators (cryptochromes acting in blue- and red-light induction and phyA acting in red-light induction), while up-regulation of *AtCLH2* is mediated mainly by cryptochromes and modulated by phototropins and phyB. The roles of *AtCLH1* and *AtCLH2* products are unclear but the fundamental differences in the light regulation of their transcription suggest that these enzymes play different roles in the metabolism of Chl.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds: The wild-type Columbia and phytochrome mutants of Arabidopsis (*Arabidopsis thaliana*; *phyA*, *phyB*, and *phyAphyB*) were obtained from the Nottingham Arabidopsis Stock Centre. The seeds of cryptochrome mutants (*cry1*, *cry2*, and *cry1cry2*) of Arabidopsis were a kind gift from Chentao Lin, Department of Biological Sciences, Lab for Molecular Biology, University of Illinois, Chicago. Phototropin mutants (*phot1* and *phot2*) were donated by Anthony R. Cashmore (Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia), and the double mutant *phot1/phot2* by Jose Jarillo, Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain. The phytochrome mutants were Landsberg *erecta* background while the cryptochrome and phototropin mutants were background Columbia.

All plants were grown in individual Jiffy pots in a growth chamber (Sanyo MLR 350H) under a 10-h light/14-h dark photoperiod with a photosynthetic photon flux density of 80 to 100 μmol m^{-2} s^{-1} (fluorescent lamps Sanyo FL40ss.W/37) at 23°C in 80% relative humidity.

Light Treatment

In all experiments 4- to 6-week-old plants were used. The dark adaptation lasted overnight. Individual leaves of dark-adapted plants were irradiated for 3 h with blue, red (both 40 μmol m^{-2} s^{-1}), or white (120 μmol m^{-2} s^{-1}) light. The onset of irradiation was at 10 AM, i.e. the time when the illumination would start in the growth chamber. White light was obtained from Philips Master TL-D 36W/840, Osram L 36W/77 FluorA. Blue and red light was obtained from LEDs ELEX-5AA0-D00 (Edison Opto Corp.) using plastic foil filters. The maxima of the blue and red light were at 457 ± 10 nm and 627 ± 20 nm, respectively. The nonirradiated leaves were used as a dark control.

Senescence was induced by darkening individual attached leaves of Arabidopsis wild type grown in a standard photoperiod, wrapping them in black paper. After 1 to 5 d, at 1 PM each day, the paper was removed and the leaves were illuminated with white light for 3 h. As a control, darkened but

nonirradiated leaves were used. The collected material was frozen in liquid nitrogen and used for RNA isolation.

DCMU Treatment and Chl Fluorescence Measurements

To inhibit photosynthesis, whole 5-week-old plants were sprayed either with 0.4% dimethyl sulfoxide (DMSO) containing 200 μM DCMU (Diuron, Sigma-Aldrich) or with 0.4% DMSO (control), then wrapped in plastic foil and incubated overnight in darkness. To estimate the degree of photosynthesis inhibition, measurements of Chl fluorescence were performed. The quantum yield of energy conversion in PSII was imaged in whole plants using an Open FluorCam FC 800-O/1010 imaging fluorometer (Photon Systems Instruments). In the dark-adapted plants the basal fluorescence (F_0) was recorded for 5 s, followed by a 10-s measurement of fluorescence (F_m) in the presence of red actinic light (150 μmol m^{-2} s^{-1}), and mean quantum efficiencies of PSII for individual leaves were calculated. To estimate the levels of mRNA, leaves treated with DMSO or DCMU in DMSO, were divided into two parts. One part of the leaves was harvested immediately after the measurements of Chl fluorescence and used as a dark control; the other part was first irradiated for 3 h with white light and then collected. Dark-adapted and/or illuminated leaves were immediately frozen in liquid nitrogen and used for RNA isolation.

RNA Isolation and Real-Time RT-PCR

Total RNA from individual leaves was isolated using TRI reagent (Bio-Chemika) according to the manufacturer's instructions. For the experiments with photoreceptor mutants, single leaves from at least 10 plants were pooled and RNA was isolated using a spectrum plant total RNA kit (Sigma-Aldrich). The concentration of RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and RNA integrity was verified by electrophoresis on 2% agarose gel. The cDNA was synthesized from 1 μg of total RNA with random hexamer primers using a RevertAid M-MuLV reverse transcriptase kit (Fermentas UAB). Real-time RT-PCR was performed with a SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and a real-time thermal cycler Corbett Rotor-Gene 6000 (Corbett Research). In a single reaction, the cDNA corresponding to 50 ng of RNA was taken and all reactions were run in duplicate (triplicate for photoreceptor mutants experiments). The primers used in this work are listed in Supplemental Table S1. The selection of reference genes (*UBC*, *SAND*, and *PDF*) and corresponding primer sequences were obtained according to Czechowski et al. (2005). The primer sets for both enzymes were designed to flank introns. The specificity of the products was analyzed by electrophoresis on 2% agarose gel in a Tris-acetate-EDTA buffer and confirmed at the end of each run by melt curve analysis. The relative levels of mRNA of each gene in the samples were determined using the mean value of Ct for dark-adapted leaves of Arabidopsis wild type in a given run as a reference. The normalization of the *AtCLH1* and *AtCLH2* mRNA levels was performed using normalization factors based on the reference gene levels calculated by geNorm v3.4 (Vandesompele et al., 2002).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. List of primer sequences used.

LITERATURE CITED

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