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Original Paper

Regulation of xanthophyll cycle pool size in response to high light irradiance in *Arabidopsis*

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Abstract The xanthophyll cycle is known to play a key photoprotective role in plants. While it has been demonstrated that the pool size of xanthophyll cycle pigments (VAZ) increases during acclimation to high light (HL) intensity, the associated regulatory mechanism remains largely unknown. Since the redox state of Plastoquinone (PQ) is thought to influence the expression of photosynthesis-related genes, we addressed the possibility that the redox state of PQ affects the regulation of carotenoid synthesis. To do this, we used a photoautotrophic cell culture of *Arabidopsis*, that can grow stably in sugar-free medium, and regulated the redox state by adding the electron transport inhibitors, DCMU or 2,5-dibromo-3-methyl-6-isopropyl benzoquinone (DBMIB) to the cells. We then analyzed their effects on VAZ accumulation and the expression of genes encoding carotenoid biosynthesis enzymes. The VAZ pool size and the transcript levels of β-carotene hydroxylase genes (*Chy1*, *Chy2*, *CYP97A3* [*Lut5*]) were higher under HL than under normal conditions; however, DCMU treatment partially blocked these effects. In contrast, DBMIB treatment increased VAZ accumulation and transcription of these genes without HL irradiation to a certain extent. Based on these results, we propose that the redox state of PQ is one of the regulator of the pool size of VAZ. The expression of *LcyB* encoding lycopene β-cyclase was also clearly up-regulated by HL, but not affected by PQ redox. In addition, using mutants of the β-carotene hydroxylase genes, we identified *Chy1* as the gene that contributed most to the increase in VAZ pool size by HL.

Key words: *Arabidopsis*, carotene hydroxylase, plastid signal, photoautotrophic culture, xanthophyll cycle.

Light is essential for plants, but when leaves are exposed to a HL intensity that exceeds the quantity required to drive photosynthesis, the excess excitation energy can accumulate and, as a consequence, damage the photosynthetic apparatus. Plants protect themselves from such damage by thermal dissipation of excess excitation energy within the photochemical system, resulting in non-photochemical quenching (NPQ) (de Bianchi et al. 2010; Holt et al. 2004).

While the precise mechanism is not understood, the induction of NPQ is known to occur primarily in response to: (i) the conformational change of the light-harvesting complex (LHC) that is induced by the acidification of the thylakoid lumen (Horton et al. 1996); (ii) the xanthophyll zeaxanthin (Z), which bound to the LHC (Avenson et al. 2008; Holt et al. 2005); and (iii) the PsbS protein, a member of the LHC superfamily (Barros et al. 2009; Li et al. 2000). During HL irradiance, the xanthophyll cycle is activated, involving the formation of zeaxanthin via antheraxanthin (A) through de-epoxidation of violaxanthin (V) (Figure 1). Violaxanthin de-epoxidase is activated upon acidification of the thylakoid lumen in response to HL (Pfündel and Dilley 1993). It has been reported that the major role of Z in NPQ is to deactivate excited singlet chlorophyll (^{1}Chl*) (Havaux and Niyogi 1999; Müller et al. 2001) and to modulate Chl triplet state (^{3}Chl*) yield (Dall'Osto et al. 2012), but Z also functions as an antioxidant in the lipid phase of the membrane (Johnson et al. 2007). An increase in the total pool size of xanthophyll cycle pigments (VAZ) during long-term acclimation to HL intensity conditions is known to occur in a wide range of plant species (Demmig-Adams and Adams 1992b; Thayer and Björkman 1990) and chilling (Koroleva et al. 2000) and drought stress (Baraldi et al. 2008; Demmig et al. 1988) can also affect the VAZ pool size. Overexpression of the β-carotene hydroxylase gene, *Chy1*, which synthesizes zeaxanthin from β-carotene, was reported to result in a two-fold increase in the size of VAZ and enhanced the tolerance to HL and high temperature (Davison et al. 2002). Finally, control of the VAZ pool is also required to optimize the kinetics of NPQ (Johnson

Abbreviations: A, antheraxanthin; ETR, electron transport rate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl benzoquinone; DMSO, dimethylsulfoxide; HL, high light; NAA, 1-naphthaleneacetic acid; NPQ, non-photochemical quenching; PA, photoautotrophic; PAR, photosynthetic active radiation; PM, photomixotrophic; Φ_{PSII}, PSII quantum yield; PQ, plastoquinone; qP, photochemical quenching; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; V, violaxanthin; VAZ, xanthophyll cycle pigments; Z, zeaxanthin

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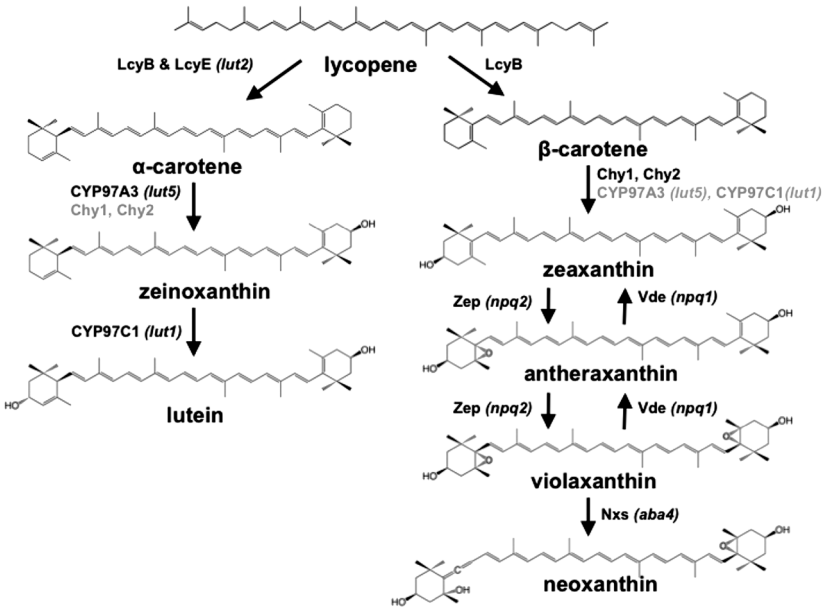

Figure 1. The carotenoid biosynthetic pathway of *Arabidopsis thaliana*. The names of the enzymes controlling each step are indicated. When applicable, mutant names are given in parentheses. The genes encoding minor activities are shown in gray. LcyB: lycopene β-cyclase; LcyE: lycopene ε-cyclase; Chy1 and Chy2: β-carotene hydroxylase; CYP97A3(*lut5*): carotene β-ring hydroxylase; CYP97C1(*lut1*): zeinoxanthin ε-ring hydroxylase; Zep: zeaxanthin epoxidase; Vde: violaxanthin de-epoxidase; Nxs: neoxanthin synthase.

et al. 2008). Despite the seemingly important functions of an increased VAZ pool, very little is known about the underlying regulatory mechanisms. The VAZ pool size in transgenic potato with reduced photosynthetic capacity was always higher than that of wild-type plants at all light intensities (Bilger et al. 1995), while the VAZ pool size in duckweed was regulated not only by light itself, but also by a mechanism triggered by a factor associated with the de-epoxidation state of the xanthophyll cycle (García-Plazaola et al. 2002).

Changes in the functional or metabolic status of plastids are known to influence the expression of nuclear genes encoding plastid proteins, and have become collectively known as 'plastid signals' (Chi et al. 2013; Inaba 2010; Leister 2012; Pfannschmidt 2010). Such signals include the redox status of the photosynthetic electron transport chain (Baier and Dietz 2005; Fey et al. 2005), the expression of plastid genes, reactive oxygen species (ROS) (Galvez-Valdivieso and Mullineaux 2010; Lee et al. 2007; Maruta et al. 2012), and tetrapyrrole compounds (Strand et al. 2003). Since all genes involved in carotenoid synthesis are encoded in the nucleus, it is assumed that certain signals induced by HL irradiation are transmitted to the nucleus where they modulate the expression of these genes.

The aim of this current research was to clarify how HL intensity signals increase the VAZ pool during acclimation to HL conditions by investigating the effect of light intensity and the redox state of the photosynthetic electron transport chain on xanthophyll biosynthesis. We used cultured green cells of *Arabidopsis* as a model for mesophyll cells. Cell suspension cultures of higher plants provide a useful system for photosynthetic research because the structure and function of the photosynthetic apparatus can be studied at the single cell level under controlled environmental conditions, such as light, temperature, osmotic pressure and nutrition. Although most cultured cells are not suitable for studies of photosynthesis because they do not have developed chloroplasts and require media containing a carbon source, photoautotrophic (PA) suspension cultures from different plant species have been established that are able to grow using CO_2 fixed by photosynthesis. Consequently, PA cells can be very useful in the characterization of photosynthesis related metabolism, production of secondary metabolites and environmental stress responses (Hampp et al. 2012; Roitsch and Sinha 2002; Sato 2013). We selected *Arabidopsis* cells exhibiting a strong green color to establish PA cells, since they have fully developed chloroplasts and can grow stably in light without the addition of carbon sources. We previously reported that the growth of PA tobacco cells was severely suppressed by herbicides, such as atrazine and paraquat, which primarily inhibit or disturb photosynthetic processes, compared to photomixotrophic and heterotrophic cells (Sato et al. 1987). Moreover, PA tobacco cells have better developed chloroplasts and higher photosynthetic activities than the photomixotrophic cells (Takeda et al. 1989; Takeda et al. 1999). Here, we established a PA cell suspension culture of *Arabidopsis* to study regulation of the xanthophyll cycle pool size by light and chloroplast

redox signals.

Materials and methods

Induction of Arabidopsis calli

Seeds of *Arabidopsis thaliana* L. Heynh. ecotype Columbia *gl-1* were surface sterilized by sequential treatment with 70% ethanol and 2% sodium hypochlorite. After washing five times with sterilized water, the seeds were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 1.5% (w/v) sucrose. After germination, hypocotyls were excised and sub-cultured on MS media containing various concentrations of phytohormones (10^{-6}–10^{-4} M 1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4-D) and 10^{-7}–10^{-5} M benzyl adenine), 2% sucrose and 0.25% gelrite. Four weeks after incubation under continuous white fluorescent light of 100 μmol photons m^{-2} s^{-1} at 25°C, the greenest and fastest proliferating calli were identified on medium containing 10^{-5} M NAA and 10^{-6} M benzyl adenine, and those calli were sub-cultured in liquid MS medium with the same phytohormone content.

Development of the photoautotrophic culture

Photomixotrophically cultured *Arabidopsis* cells were maintained in media containing 2% sucrose on a gyratory shaker (100 rpm) and sub-cultured every two weeks. Photoautotrophic (PA) suspension cells were initiated from photomixotrophic cells by inoculating them in the same media, but with 1% sucrose, in air enriched with CO_2 (1–2%) as previously described (Yamada and Sato 1978), with the modification of using two-tier flasks (Hüsemann and Barz 1977). Cells were transferred to fresh media containing 0.5% sucrose after three to four weeks and subsequently moved to media containing no sucrose in CO_2-enriched air. They were transferred to fresh sugar-free media when the cells increased by two-three times. This cycle was repeated until stably growing cells were obtained. The PA *Arabidopsis* cells studied here were grown under PA conditions for about 12 months before analysis. PA and PM cells were sub-cultured every three or two weeks, respectively. Both types of cells were cultured at 25°C with rotary shaking (100 rpm) under continuous white light (100 μmol m^{-2} s^{-1}).

T-DNA mutants

The T-DNA insertion mutants used in this study were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, USA. The corresponding line names of the β-carotene hydroxylase alleles are: *chy1* (AT4g25700), SALK_056859C; *chy2* (AT5G52570), SALK_044659C; *lut5* (AT1G31800), SALK_116660. Homologous mutants were identified by PCR analyses with the primer pairs flanking the T-DNA insertion site and primers from each β-carotene hydroxylase sequence (Table S1). For analysis of the mutants, surface sterilized seeds were plated on half-strength MS media containing 1.5% (w/v) sucrose, incubated for 3 days at 4°C in the dark, and grown for 3–4 weeks in a growth chamber at 23°C under 10 h light/14 h dark conditions (100 μmol m^{-2} s^{-1}).

Pigment analyses

Leaves and cell samples were frozen in liquid nitrogen and ground in cold 85% acetone, centrifuged and washed twice with 100% acetone. Following filtration through the syringe filter, the extracts were immediately analyzed by HPLC, as described previously (Masamoto et al. 1993; Takeda et al. 1996).

Measurement of plastoquinone

Arabidopsis cells were frozen in liquid nitrogen and homogenized in cold acetone, centrifuged and washed once with acetone. The extracts were then filtered and immediately analyzed by HPLC. HPLC measurements were performed using a JASCO (JASCO, Tokyo, Japan) pump (PU-980) and UV–VIS detector (UV-970), with a C18 reverse-phase column (ODS-AL313; YMC, Kyoto, Japan), isocratic solvent system (1 : 1, ethanol : methanol, v/v), with absorption detection at 255 nm (Kruk and Karpinski 2006). Concentrations of oxidized PQ were determined from the area of the corresponding peak in the chromatograms and the peak of a standard oxidized PQ solution. The reduced PQ (PQH_2) in the same sample was fully oxidized by adding $FeCl_3$ and analyzed by HPLC to determine the total plastoquinone ($PQ+PQH_2$) content. The ratio of reduced to oxidized PQ (PQH_2/PQ) was then calculated. The PQ standard was kindly donated by Dr. Shibata of Nagaoka National College of Technology.

Measurements of chlorophyll fluorescence

Fluorescence emission from cells was measured using a PAM fluorometer (Walz, Effeltrich, Germany). Cells were dark-adapted for 30–60 min prior to analysis then placed in the suspension cuvette with 20 mM Tricine–NaOH (pH 7.8) buffer containing 5 mM $NaHCO_3$. The fluorescence parameters ware determined as follows (van Kooten and Snel 1990). At the start of each experiment, saturating pulses (1.0 s) were applied to determine Fm (maximum fluorescence after dark-adaptation) values. Actinic LED light and measuring LED light were filtered through a short-pass filter ($\lambda<670$ nm). Fo (minimum fluorescence after dark-adaptation) values were calculated as the fluorescence yield when the actinic light was turned off, and Ft as the fluorescence yield during actinic illumination. Fo′ (minimum fluorescence in the light-adapted state) was measured in the presence of far-red light after turning off the actinic light, with Fm′ representing the maximum fluorescence during actinic illumination.

Light curves of the fluorescence parameters were determined as follows. Actinic light was increased at five-min intervals and fluorescence was measured at each step. Efficiency of PSII photochemistry (Φ_{PSII}) was calculated as (Fm′–Ft)/Fm′; relative PSII electron transport rate (ETR) as $\Phi_{PSII}\times PAR\times 0.84\times 0.5$; gross non-photochemical quenching (NPQ) as (Fm–Fm′)/Fm′; and the fraction of Q_A in the reduced state (1-qP) as (Ft–Fo′)/

(Fm′–Fo′).

Analysis of gene expression

Total RNA was extracted from the cultured cells and plants using an RNeasy Plant Mini kit (Qiagen GmbH., Hilden, Germany). Five micrograms of total RNA was used as a template, and first-strand cDNA synthesis was performed using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Otsu, Japan), according to the manufacturer's instructions. Subsequent amplification of the first-strand cDNA was performed using Taq DNA polymerase (New England Biolabs, Ipswich, MA) and a thermal cycler (Gene Atlas 322, ASTEC, Fukuoka, Japan). Table S1 lists the primers used.

Results

Establishment of a photoautotrophic (PA) Arabidopsis cell culture

Approximately 100 lines of calli from sterile hypocotyls of *Arabidopsis* seedlings were induced on solid media containing sucrose. The greenest calli were selected, incubated photomixotrophically in liquid media containing 2% sucrose, and sub-cultured every two weeks. After approximately six months, the cells divided at an almost constant rate, generating a 10 fold increase in the amount of material in fresh weight every two weeks. These photomixotrophic cells were then sub-cultured in media containing a lower concentration of sugar and placed in CO_2 enriched air. Finally, a PA cell line was established that was able to grow stably in sugar-free media, albeit at a slower rate (about 4-5-fold in three weeks). Table 1 shows the chloroplast-related characteristics of photomixotrophic (PM) and photoautotrophic (PA) *Arabidopsis* cells. Apparently, the PA cells had more chloroplasts per cell, and more chlorophyll and carotenoid on a fresh weight basis than the PM cells, although the values were still very low compared to those of leaves. In addition, the Chl/Carotenoid ratio of PA cells is similar to the ratio of leaves more than PM cells, while the Chl *a*/*b* ratio in PA cells is higher than PM cells and leaves.

Adaptation of photoautotrophically cultured cells to high light (HL) intensity conditions

The growth rate of *Arabidopsis* PA cells was the highest under 100 μmol photons m^{-2} s^{-1}, which we will refer to as the growth light (GL) intensity (Figure 2A). When the light intensity was higher than 100 μmol photons m^{-2} s^{-1}, the Chl content decreased (Figure 2B) while the Chl content in plant leaves did not change (Supplementary Figure S1A), indicating that the PA cells were more sensitive to light than plant leaves. Regarding the Chl *a*/*b* ratio, the values in the PA cells were lower in high light intensities (Figure 2C) while the ratio in leaves was higher under high light (Supplementary Figure S1B). On the other hand, the content of certain carotenoids in the PA cells increased at light intensities >150 μmol photons m^{-2} s^{-1}: this was most evident for lutein and

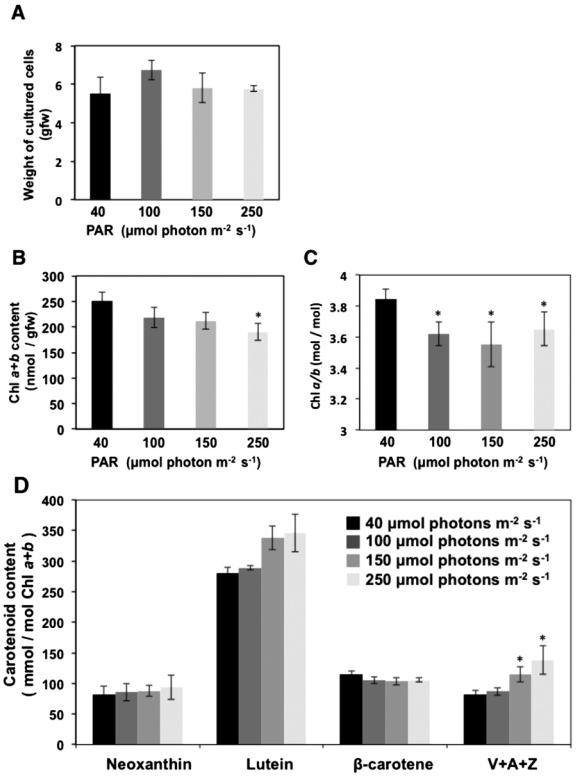

Figure 2. Effects of light intensity on growth (A), chlorophyll content (B), chlorophyll *a*/*b* ratio (B) and carotenoid content (D) in cultured *Arabidopsis* cells. Two-week-old cultured *Arabidopsis* cells grown under 100 μmol photons m^{-2} s^{-1} were cultured under different light intensities for one week then subjected to analysis. Error bars indicate the SE from three individual experiments. An asterisk indicates a significant difference from the sample of 40 μmol photons m^{-2} s^{-1} by Student's *t*-test ($p<0.05$).

Table 1. Number of chloroplasts and pigments in photomixotrophically and Photoautotrophically cultured cells and leaves of *Arabidopsis*.

	Photomixotroph	Photoautotroph	Leaves
Number of chloroplasts/cell	14.3±2.5	51.3±7.7	76*–121**
Chl *a*+*b* content (nmol gFW^{-1})	45.7±7.8	152.3±18.2	1513±224
Chl *a*/*b* (ratio)	2.49±0.17	3.47±0.20	2.49±0.07
Carotenoid content (nmol gFW^{-1})	33.8±5.2	75.4±8.4	326±48.3
Chl/Carotenoid ratio	1.35±0.37	2.02±0.42	4.64±1.61

*Kinsman and Pyke, 1998, **Pyke and Leech, 1992

the pool size of VAZ (Figure 2D). Previous studies have reported an increase in VAZ under high light intensity conditions in the leaves of various plants (Demmig-Adams and Adams 1992b; Thayer and Björkman 1990). Based on these studies and our initial investigations, we defined 40 μmol photons $m^{-2} s^{-1}$ as low light (LL), 100 μmol photons $m^{-2} s^{-1}$ as growth light (GL), and 250 μmol photons $m^{-2} s^{-1}$ as high light (HL) in subsequent experiments.

Figure 3 shows the relative rate of the PSII electron transport (ETR), the efficiency of PSII photochemistry (Φ_{PSII}), steady state values of gross NPQ and the estimated reduction state of the first stable PSII electron acceptor Q_A (1-qP) during exposure to an increasing amount of photosynthetic active radiation (PAR) in the *Arabidopsis* PA cells. The relative rate of the PSII electron transport of PA HL cells was higher than that of PA LL cells at elevated PARs; that is, HL cells resembled the HL acclimated leaves (Figure 3A). This is because the efficiency of PSII photochemistry was lower in HL cells than LL cells at PAR levels <210 μmol photons $m^{-2} s^{-1}$, but higher than in LL cells at elevated PARs (Figure 3B). HL cells also exhibited a higher steady-state NPQ than LL cells at a PAR >500 μmol photons $m^{-2} s^{-1}$, indicating a strong non-photochemical quenching ability of HL cells (Figure 3C). Consistent with their ability to dissipate excess light energy, HL cells also showed a lower reduction state of Q_A than LL cells (Figure 3D). Finally, the induction and relaxation of NPQ was measured in LL and HL (Figure 3E) cells, and the maximum NPQ value in HL cells during exposure to HL intensity was found to be higher than that of LL cells, although it was lower than that of leaves (Supplementary Figure S2). Upon subsequent darkening, the NPQ of HL cells relaxed more quickly compared to LL cells, indicating that HL cells have larger energy quenching (qE) than LL cells.

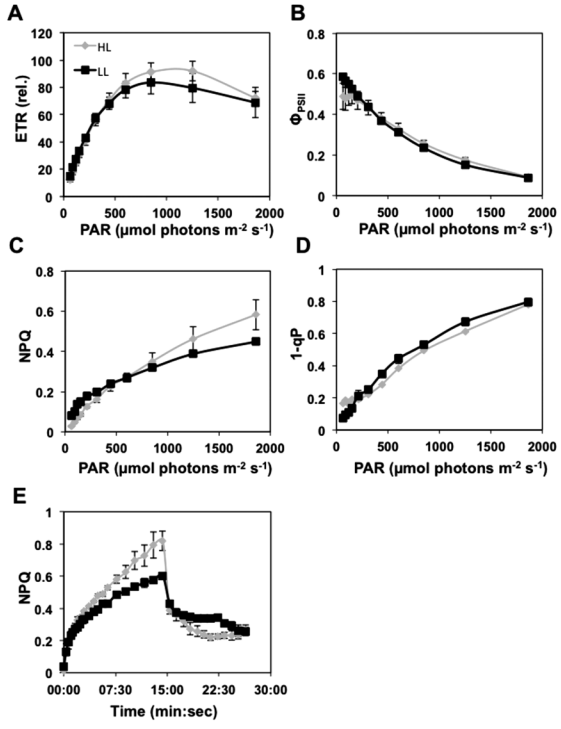

Figure 3. Light dependence of steady-state chlorophyll fluorescence parameters in *Arabidopsis* cells grown in low light (LL) and high light (HL). Each point represents the mean of three measurements and error bars indicate the SE. (A) The relative PSII electron transport rate (ETR), calculated as $\Phi_{PSII} \times PAR \times 0.84 \times 0.5$. (B) Efficiency of PSII photochemistry (Φ_{PSII}), calculated as (Ft–Fo′)/(Fm′–Fo′) during light. (C) Gross non-photochemical quenching (NPQ). (D) The estimated reduction state of Q_A(1-qP). The fraction of Q_A in a reduced state was estimated as (Ft–Fo′)/(Fm′–Fo′). (E) Time courses of induction and relaxation of NPQ. Actinic light (1860 μmol photons $m^{-2} s^{-1}$) was switched on at time zero and switched off after 15 min.

Effects of changes in the redox state of plastoquinone caused by electron transport inhibitors

Figure 2D shows the increase in pool size of VAZ in *Arabidopsis* PA cells during an increase in light intensity. Changes in light intensity affect chloroplast functions by altering the redox state of electron transport intermediates in the chloroplast thylakoids, and the expression of certain nuclear encoded genes related to photosynthesis is known to be regulated by these plastid-derived signals. It is reported that sub-lethal concentrations of DCMU, which blocks the electron transport from PSII to plastoquinone (PQ), causing the latter to transition to an oxidized state, mimic the effects of acclimation to LL (Escoubas et al. 1995; Masuda et al. 2003; Shimizu et al. 2010). The mode of action of DBMIB, on the other hand, is to reduce the rate of PQ oxidation by competitively binding to the cytochrome b_6/f complex, resulting in PQ transitioning to a reduced state, as is seen in HL cells. We used DCMU and DBMIB to alter the redox state of PQ in *Arabidopsis* cells by adding sub-lethal concentrations to the culture media, thereby inducing an oxidized or a reduced, respectively. In order to verify that the redox state of PQ was altered, we evaluated the expression of the *PsaA* and *PsbA* genes. Expression of *PsaA*, which encodes an apoprotein from the PS-I reaction center, increases when the PQ is reduced, while expression of *PsbA*, which encodes the D1 protein of the PS-II reaction center, increases when the PQ is oxidized, although to a lesser extent (Pfannschmidt et al. 1999; Shimizu et al. 2010). Transcript levels of *PsaA* increased under HL conditions, but never in the presence of DCMU, indicating that DCMU mimicked the effects of LL (Figure 4A, B). In contrast, DBMIB resulted in higher *PsaA* transcript under GL conditions, confirming that DBMIB mimics the effects of HL intensity. Further evidence of the opposite regulatory pattern was observed for *PsbA*. The rate of accumulation of the reduced form of PQ (PQH_2) was also determined by extracting and analyzing PQ abundance using high pressure liquid

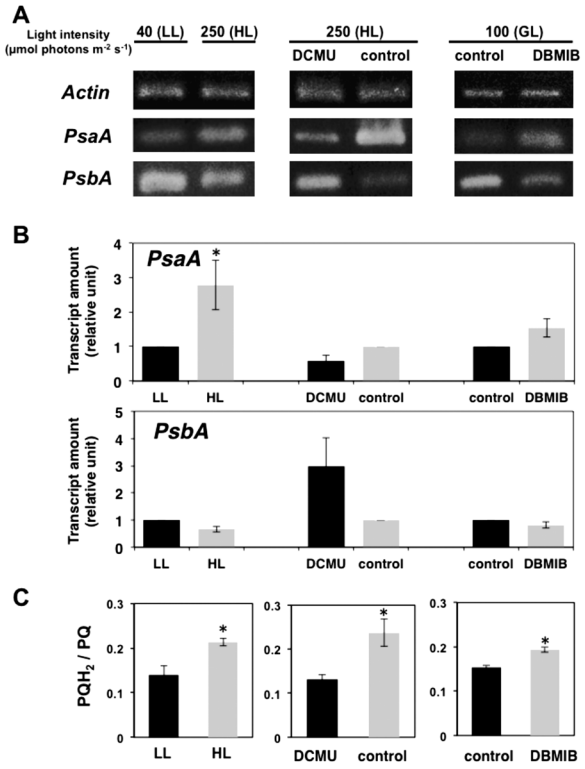

Figure 4. Effect of high light intensity and electron transport inhibitors (DCMU and DBMIB) on the transcript levels of genes for PS-I (*PsaA*) and PS-II (*PsbA*), and the redox state of plastoquinone (PQ). One- to two-week-old cultured *Arabidopsis* cells grown under 100 μmol photons m^{-2} s^{-1} (growth light) were cultured under: 1) 40 μmol photons m^{-2} s^{-1} (low light, LL) or 250 μmol photons m^{-2} s^{-1} (high light, HL); 2) media containing 3×10^{-7} M DCMU under HL conditions; 3) media containing 10^{-7} M DBMIB under 100 μmol photons m^{-2} s^{-1} for 48 h. Subsequently, cells were subjected to either a gene expression analysis using RT-PCR or determination of PQ contents by HPLC. (A) Electrophoretogram of DNAs amplified by RT-PCR. Levels of cDNA for PCR were normalized to that of *Actin* cDNA in the samples. (B) Intensity of the respective band measured upon a densitometric scan. Values were normalized to that of LL or control in each experiment. (C) The ratio of plastohydroquinone (PQH_2) to PQ in the *Arabidopsis* cells. Data are means ±SE from three experiments. An asterisk indicates a significant difference from LL or control by Student's *t*-test ($p<0.05$).

chromatography (HPLC) (Kruk and Karpinski 2006). Figure 4C shows that PQH_2/PQ was higher in HL cells than LL cells, with a lower ratio of PQH_2 in cells treated with DCMU compared to control cells, while DBMIB treatment increased this ratio. We were thus able to confirm the modulation of the redox status of PQ in these *Arabidopsis* cells using DCMU and DBMIB.

Changes in the pool size of xanthophyll cycle pigments due to modulation of the redox status of PQ

Figure 5 shows the changes in pool size of VAZ induced by DCMU and DBMIB. *Arabidopsis* cells treated with DCMU, which consequently had a more oxidative PQ redox than cells without DCMU (control), had a lower

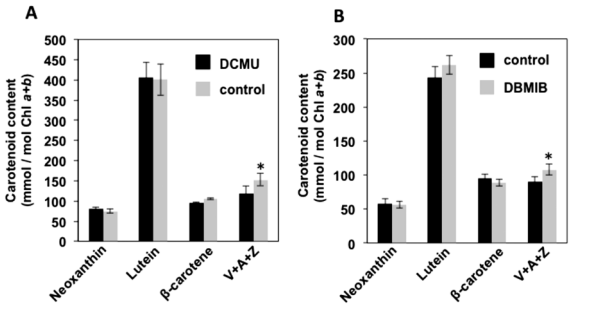

Figure 5. Effects of photosynthetic electron transport inhibitors on the level of carotenoids in cultured *Arabidopsis* cells. Conditions were as described in the legend of Figure 4. (A) DCMU was added to a final concentration of 3×10^{-7} M under HL conditions and (B) DBMIB to a final concentration of 10^{-7} M under GL conditions. Error bars indicate the SE from three different experiments. An asterisk indicates a significant difference from the control by Student's *t*-test ($p<0.05$).

VAZ content than control cells (Figure 5A). In contrast, the addition of DBMIB resulted in an increase in VAZ, as was observed when cells were incubated under the HL condition (Figure 5B). In addition, neither DCMU nor DBMIB influenced chlorophyll composition much (Supplementary Figure S4). These changes in pigment content suggest that PQ in a reduced state promotes the synthesis of xanthophyll cycle pigments in *Arabidopsis* cells.

Changes in the expression of genes for carotenoid synthesis due to modulation of the redox status of PQ

One explanation for the fluctuations in carotenoid content following changes in light intensity and treatment with the electron transport inhibitors was that these treatments altered the expression of carotenoid synthesizing genes; a hypothesis that we investigated by semi-quantitative RT-PCR. The expression patterns of *LcyE* (*Lut2*) encoding lycopene ε-cyclase, *LcyB* encoding lycopene β-cyclase, *Chy1, Chy2* and *CYP97A3* (*Lut5*), which encode β-carotene hydroxylases, and *CYP97C1* (*Lut1*), which encodes an zeinoxanthin ε-ring hydroxylase (Figure 1) were all analyzed. It was found that the expression of *LcyB, Chy1, Chy2* and *CYP97A3* was higher in HL cells than in LL cells, suggesting that an increase in expression of these genes contributed to an increase in xanthophyll cycle pigments in HL cells (Figure 6A, B). While DCMU treatment repressed the increased expression of three β-carotene hydroxylase genes (*Chy1, Chy2* and *CYP97A3*) under HL conditions, DBMIB induced their expression, again mimicking either LL or HL, respectively. The effects of DCMU and DBMIB also suggest that the increase in expression of *Chy1, Chy2* and *CYP97A3* under HL illumination could be promoted by the reduced state of PQ. On the other hand, the transcript levels of *CYP97C1*(*Lut1)* transcripts, another hydroxylase gene required for ε-ring hydroxylation,

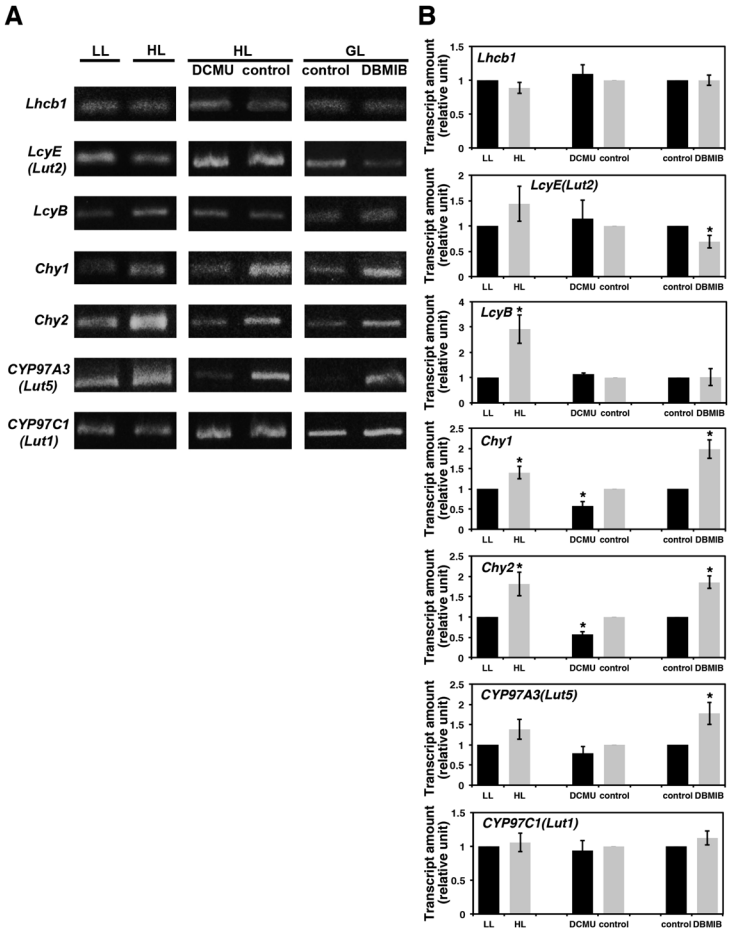

Figure 6. Effect of high light intensity and electron transport inhibitors (DCMU and DBMIB) on the transcript levels of genes for Lhcb1, carotenoids and chlorophyll biosynthesis. Conditions were as described in the legend of Figure 4. (A) Electrophoretogram of DNAs amplified by RT-PCR. Levels of cDNA for PCR were normalized to that of *Actin* cDNA (Figure 4) in the samples. (B) Intensity of the respective band measured upon a densitometric scan. Values were normalized to that of LL or control in each experiment. Data are means ±SE from three to five experiments. An asterisk indicates a significant difference from LL or control by Student's *t*-test ($p<0.05$).

were not affected by the change in light intensity or the electron transport inhibitors, and neither were those of *Lhcb1*, the encoded protein of which contains binding sites for xanthophylls.

Xanthophyll cycle pigment content in β-carotene hydroxylase mutants

To determine which of the three β-carotene hydroxylases (*Chy1, Chy2* and *CYP97A3*), contributed most to the increase in size of xanthophyll cycle pigments, we investigated the change in pigment content induced by HL irradiation using T-DNA insertion mutants for genes encoding the β-carotene hydroxylases. All T-DNA insertions disrupted exon sequences, segregated as single Mendelian loci and resulted in no detectable accumulation of transcripts corresponding to the mutated genes (Supplementary figure S3); yet none of the mutants displayed a visible phenotype under laboratory conditions. The changes in pigment content in the mutants after HL irradiation indicated that in *chy2* and *lut5*, the VAZ content increased about 1.3- and 1.6-fold, respectively, and the lutein content also increased, comparable to the wild type (WT) (Figure 7). However, in *chy1*, the VAZ and lutein content showed no increase and the β-carotene decreased (Figure 7). suggesting that *Chy1* is primarily involved in increasing the pool size of carotenoids after HL irradiation.

Discussion

Arabidopsis photoautotrophic culture as a model system for studying the excess light acclimation process

In this study, we established an *Arabidopsis* PA culture for use in studying the process of acclimation to HL irradiation. Our results showed that the cultured cells acclimated to HL intensity, increasing the efficiency of PSII photochemistry, NPQ and the pool size of xanthophyll cycle pigments under HL intensity (Figure 2D and Figure 3). While the Chl content in HL cells decreased >20% compared with LL cells (Figure 2B), the Chl content in leaves did not decrease under HL

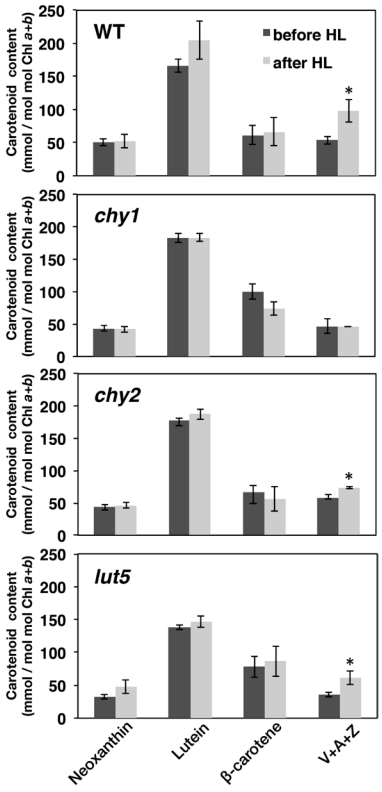

Figure 7. Effect of high light intensity on the levels of carotenoids in the leaves of wild-type (WT), *chy1*, *chy2* and *lut5*. Two-week-old *Arabidopsis* seedlings grown under 100 μmol photons $m^{-2} s^{-1}$ were exposed to a PAR of 250 μmol photons $m^{-2} s^{-1}$ (HL) for 48 h then harvested for pigment analyses. An asterisk indicates a significant difference between the value before and after the HL treatment by Student's *t*-test ($p<0.05$).

intensity (Supplementary Figure S1A). This suggests that the PA cells were more sensitive to HL than plant leaves, possibly because the PA cells could not increase their electron transport rate sufficiently for adaptation to HL conditions. Since the chloroplast number per cell and pigment content of the PA cells were much higher than those of the PM cells, but not as high as plant leaves (Table 1), and the maximum electron transport rate (ETR) of the HL grown PA cells (Figure 3A) was lower than that of HL grown plant leaves (Supplementary Figure S2A), it seems the PA cells have more excess excitation energy than whole plants. The Chl *a*/*b* ratio in HL grown green leaves, including *Arabidopsis* (Supplementary Figure S2B), generally higher because of the reduction of antenna size, but Chl *a*/*b* ratio in HL cells was lower than LL cells (Figure 2C). This result could have been caused by the reduction of Chl *a* during high light irradiation. The analysis of antenna size and PSI/PSII ratio in PA cells are necessary.

The relative ratio of carotenoids to Chl *a*+*b* was much higher in the PA cells (Figure 2D) than in the leaves (Supplementary Figure S1C), as was previously reported for cultured green cells of tobacco and tobacco leaves (Takeda et al. 1996). One explanation for this might be that the cultured cells need more carotenoid to dissipate the excess excitation energy because of their low photosynthetic electron transport rate. During acclimation to HL irradiation, the *Arabidopsis* PA cells showed an increase in VAZ (Figure 2D) as observed in leaves in *Arabidopsis* (Supplementary Figure S1C) and many other plants (Demmig-Adams and Adams 1992a; Thayer and Björkman 1990), which has also been previously observed in cultured green cells of tobacco (Takeda et al. 1996), and the changes of photosynthetic functions of *Arabidopsis* cells induced by HL irradiation were also similar to those of leaves (Figure 3, Supplementary Figure S2). Based on the above results, we considered the *Arabidopsis* PA cells to be a suitable model system for mimicking mesophyll cells and for studying the HL intensity acclimation process. Hampp et al. (2012) also established an *Arabidopsis* PA cell culture and reported that the PA cells would be useful for addressing responses such as signal cascades, as well as for studying photosynthesis and other aspects of plant metabolism.

Regulatory mechanism underlying the increase in xanthophyll cycle pigments in response to excess light

Independent of light intensity, the change in the redox state of PQ altered the VAZ pool size in the PA cells (Figure 5); however, while the lutein content also increased in HL cells, treatment with DCMU or DBMIB did not result in any notable change in lutein content, suggesting that the change in PQ redox state did not regulate the content of all of the carotenoids. The HL induced change of carotenoid contents might be caused by multiple factors, such as enzyme activity, availability of the substrates or electron carriers and gene expression. The increase of Z in response to HL irradiation is known to be caused by the activation of violaxanthin de-epoxidase, which is induced by acidification of the thylakoid lumen (Pfündel and Dilley 1993). HL irradiation also changes the redox state of other electron carriers, such as ferredoxin, thioredoxin, glutathione and NADPH converting them to into reduced form, and might change the activities of enzymes involved in carotenoid synthesis. These factors might be important for the short-term response to HL irradiation, but we focused on the changes in expression of genes associated with carotenoid synthesis for the long-term acclimation. *Arabidopsis* contains two different classes of carotenoid hydroxylases: Chy1 and Chy2, non-heme di-iron enzymes similar to the bacteria CrtZ and the cyanobacteria CrtR-B enzymes, which primarily catalyze the hydroxylation of the β-ring of β-carotene (β,β-carotene); and CYP97A3 (Lut5) and CYP97C1 (Lut1), cytochrome P450 enzymes that hydroxylate the β-ring of α-carotene (ε,β-carotene) and the ε-ring of zeinoxanthin,

respectively (Figure 1). *Chy1* and *Chy*2 encode isozymes, but do not show strong co-expression, while *CYP97A3* and *CYP97C1* encode enzymes with different in vivo substrates but are also strongly co-expressed (Kim et al. 2009; Ruiz-Sola and Rodriguez-Concepcion 2012).

Figure 6 shows that HL irradiation increased the expression of *Chy1, Chy2* and *CYP97A3*, β-carotene hydroxylase genes involved in xanthophyll synthesis. In *Arabidopsis* leaves, the expression of *Chy1* and *Chy2* have been reported to increase substantially by HL treatment, while the expression of *CYP97C1* does not show any increase (Kleine et al. 2007), suggesting the similarities in gene expression patterns between the PA cells and leaves.

We further examined the possible involvement of PQ redox status in these changes in expression (Figure 6). When DCMU inhibited the electron flow from PSII to PQ, oxidizing the PQ, levels of *Chy1, Chy*2 and *CYP97A3* transcripts were less than in the control, indicating that expression of these hydroxylase genes was not up-regulated by HL irradiation in the presence of DCMU. In contrast, when DBMIB reduced the PQ, transcripts levels of all three β-carotene hydroxylase genes increased. These results suggest that a change in PQ redox induced by a change in light intensity caused a signal to be sent to the nucleus, which subsequently modulates the expression of *Chy1, Chy*2 and *CYP97A3.* However, in tobacco seedlings, an expression analysis of material treated with DCMU and DBMIB indicated the opposite pattern to be true, in that DCMU caused an increase in the transcript levels of β-carotene hydroxylase (*bhy*) and DBMIB blocked an increase of the transcript level in the light (Woitsch and Römer 2003). In this case, *bhy* mRNA levels in LL (10 μmol photon m^{-2} s^{-1}) were higher than in moderate light (100 μmol photon m^{-2} s^{-1}) during chloroplast differentiation of tobacco seedlings. The reason for these differences to light and PQ redox between *Arabidopsis* and tobacco are not clear, but we note that tobacco is more heliophilic and so a difference in light susceptibility might underlie the results: 100 μmol photon m^{-2} s^{-1} is not an excessive intensity for tobacco seedlings. On the other hand, the gene for lutein synthesis encoding zeinoxanthin ε-ring hydroxylase *CYP97C1*, which showed no change in expression due to changes in light intensity, also showed no expression changes in response to either DCMU or DBMIB, despite an increase in lutein content in HL cells. Although lutein is needed for chlorophyll triplet quenching and effective photoprotection (Dall'Osto et al. 2006), the lutein content seems to be regulated via a different mechanism to VAZ. The mRNA for *LcyB* encoding lycopene β-cyclase, a key enzyme of β-carotene production, was more abundant in HL than LL cells, as with *Chy1* and *Chy*2, but showed no change in response to DCMU or DBMIB treatment, suggesting that *LcyB* might be regulated by a plastid signal other than PQ redox status.

Although HL treatment causes substantial changes in many nuclear gene expression levels (Li et al. 2009), including *LcyB, Chy1, Chy2* and *CYP97A3*, it is not clear how excess light is sensed, or how the signal is transduced to the nucleus. Photoreceptors, such as phototropin, neochrome and cryptochrome have been reported to sense excess light and transmit signals that trigger chloroplast movement and nuclear gene expression responses. In *Arabidopsis*, cryptochromes have been shown to control many HL responsive genes (Kleine et al. 2007). Among the enzymes involved in xanthophyll synthesis, *Chy1*, *Chy2*, *CYP97A3* and *CYP97C1*, only the HL response of *Chy1* slightly decreased in *cry1*.

Excess light can also be sensed in a chloroplast-dependent manner. The changes in redox state of electron carriers, such as PQ, thioredoxin and glutathione, production of ROS, and accumulation of metabolites, including intermediates in tetrapyrrole biosynthesis, are proposed to be involved in the expression of HL responsive genes (Chi et al. 2013; Dietz and Pfannschmidt 2011; Leister 2012; Li et al. 2009). A ROS-responsive nuclear gene, *APX2*, which encodes a cytosolic ascorbate peroxidase, was shown to respond to hydrogen peroxide (H_2O_2), but also to a change in PQ redox status (Karpinski et al. 1997; Kimura et al. 2001). The expression of genes associated with xanthophyll biosynthesis was not influenced remarkably by both H_2O_2 (Maruta et al. 2012) and $^1O_2$ (Ramel et al. 2013).

Additionally, any direct effect of tetrapyrrole intermediates, such as Mg protoporphyrin IX, on xanthophyll synthesis genes is unresolved at present, although norflurazon treatment was reported to decrease the expression of many carotenoid synthesis genes, including *Chy1, Chy2, CYP97A3* and *CYP97C1* (Koussevitzky et al. 2007).

The nuclear-localized Apetala 2(AP2)-type transcription factor ABSCISIC ACID-INSENSITIVE-4 (ABI4) has been proposed to function as a node of convergence of multiple plastid retrograde signaling pathways, together with the plastid PPR GUN1 protein (Inaba 2010; Wind et al. 2013). The expression of *Chy1* and *Chy2* was repressed in *abi4* mutant (Kerchev et al. 2011) and the repression of their expression induced by norflurazon treatments was recovered in *gun1-9* mutant (Koussevitzky et al. 2007), suggesting that they are involved in GUN1-ABI4 signaling pathway.

The protein level of Lchb1, the major LHCII protein, is known to decrease in parallel with a reduction in antenna size during long-term exposure to HL intensity. In *Arabidopsis*, *Lhcb2.4* expression was repressed by the HL irradiation (Kleine et al. 2007) and regulated by PQ redox status through the binding of bZIP type transcription factors to the G-box element in the *Lhcb2.4* promoter (Shaikhali et al. 2012). We observed

that the transcript levels of *Lhcb1* in HL cultured cells did not decrease compared with those of LL cells, and neither DCMU nor DBMIB treatments resulted in a change (Figure 6). However, it is important to note that regulation of LHCII occurred at the post-transcriptional, rather than transcriptional level (Frigerio et al. 2007). In addition, Arabidopsis has several *Lhcb* genes (*Lhcb1.1–1.5*, *Lhcb2.1–2.4*, and *Lhcb3*), each of which could be regulated in different ways.

We further examined which hydroxylase gene contributed most to the increase in VAZ pool size using T-DNA insertion mutants. The results shown in Figure 7 suggest that *Chy1* might be the most important in this regard, since after HL irradiation *chy1* did not show an increased VAZ pool size, in contrast to *chy2* and *lut5*. Among the three genes, expression of *Chy1* was the highest in *Arabidopsis* rosette leaves (Ruiz-Sola and Rodriguez-Concepcion 2012). Although expression of *Chy2* was the highest in dry seeds among the three hydroxylase genes, *Chy1* expression was higher than *Chy2* in rosette leaf, suggesting that Chy2 might contribute conversion of β-carotene to β, β-xanthophylls, and subsequently to ABA in dry seeds and Chy1 might be involved in the synthesis of β, β-xanthophylls for chloroplast. Comparisons of the promoter regions of these hydroxylase genes and detailed analyses of their expression may help elucidate their functions.

This research shows for the first time that expression of the nuclear genes required for carotenoid synthesis in higher plants is regulated by the redox state of PQ in chloroplasts during HL intensity acclimation, and that, of the three β-carotene hydroxylase genes, *Chy1* is the primary participant in the HL intensity response. These insights represent an important advance in our understanding of redox signal transduction from the chloroplast to the nucleus and may be helpful in the development of HL stress-resistant plants.

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