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Phenolic compounds and carotenoids during acclimation of spring barley and its mutant *Chlorina f2* from high to low irradiance

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Abstract

We examined the dynamics of phenolic compounds (PheCs) and carotenoids (Cars) in the leaves of wild type (WT) spring barley (*Hordeum vulgare* L.) and its mutant lacking chlorophyll *b Chlorina f2* (*Clo f2*) grown from seeds at high irradiance (8 d at 1 000 μmol m^{-2} s^{-1}; HI) during 9 d of acclimation to low irradiance (50 μmol m^{-2} s^{-1}; LI). Our results show that a leaf epidermal flavonoid UV-shielding index remained rather constant after transfer of plants from HI to LI conditions and that it was significantly lower in *Clo f2* compared to WT plants. This suggests that HI pretreated plants can be well protected against excessive UV for at least 9 d, as supported also by the constant absorbance of leaf PheCs extracts in the UV-A region (at 335 nm). In contrast, absorbance in the UV-B region (at 270 nm) was reduced, particularly during the initial days of LI treatment, indicating specific changes in PheC profile. High-performance liquid chromatography of soluble PheCs revealed stable content of the major PheC saponarin during LI acclimation, whereas luteolin and feruloylquinic acid content decreased, particularly in WT plants. We also observed a pronounced decrease in Car relative content, particularly a reduction in the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin, VAZ) pool and diminution of their de-epoxidation state (DEPS) in dark-adapted leaves. As both VAZ and DEPS were higher in HI-acclimated *Clo f2* plants than they were in WT plants, the presence of a significant VAZ pool within the lipid phase of thylakoid membrane is indicated. That can contribute to antioxidant capacity particularly in *Clo f2* plants. We can therefore conclude that there is a tendency to retain the PheCs responsible for UV shielding during LI acclimation. Meanwhile, the accumulation of both PheCs and zeaxanthin serving as effective antioxidants is considerably downregulated within 9 d.

Additional key words: flavonoids, HPLC, hydroxycinnamic acids, photoprotection, UV shielding, xanthophylls.

Introduction

Sun radiation is an essential environmental factor affecting plant growth. It is both the main source of energy for photosynthesis and an important signal regulating plant metabolism and development. However, exposition of photosynthetic apparatus to excessive ultraviolet radiation (UV; particularly UV-B; 280 - 315 nm) as well as photosynthetically active radiation (PAR; 380 - 750 nm) results in overproduction of reactive oxygen species (ROS) (Asada 2006, Hideg *et al*. 2013). ROS promptly interact with proteins, lipids, and DNA, thereby causing the loss of their function and consequent disruption of such vital cellular structures as membranes and pigment-protein complexes (Demidchik 2015). The extent of resulting photooxidative damage depends on the total irradiance, duration, and spectral distribution of radiation and on the actual performance, efficiency, and acclimation capability of the plant photoprotective systems at the beginning and during

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Abbreviations: A - absorbance at respective nm; AOX - antioxidant activity; Car *x+c* - spectrophotometrically determined total carotenoid content; Car - carotenoid; Chl *a* - chlorophyll *a; Clo f2* - chlorophyll *b*-less mutant of spring barley (*Chlorina f2*); DEPS - de-epoxidation state of xanthophyll cycle pigments; HI - high irradiance; HPLC - high-performance liquid chromatography; LHC - light-harvesting complex; LI - low irradiance; PAR - photosynthetically active radiation; PheC - phenolic compound; PS II - photosystem II; ROS - reactive oxygen species; Rt - retention time; UV - ultraviolet radiation; VAZ - sum of violaxanthin, antheraxanthin, and zeaxanthin; VIS - visible light; WT - wild type.
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the stress episode.

Higher plants have developed several photoprotective mechanisms on molecular, cellular, morphological, and physiological levels to mitigate photooxidative damage. Systems of enzymatic (*e.g.,* superoxide dismutase, ascorbate peroxidase, catalase) and low-molecular (*e.g.,* ascorbate, glutathione, α-tocopherol) ROS scavengers are able to cope with existing ROS (Asada 2006, Takahashi and Badger 2011). In addition, other classes of photoprotective compounds, namely phenolic compounds (PheCs) and carotenoids (Cars), can both mitigate ROS formation and act directly as antioxidants (Havaux *et al*. 2007, Hernández *et al*. 2009, Agati *et al*. 2012).

It is well documented that UV and particularly high PAR lead to increased synthesis and accumulation of PheCs in leaves of higher plants (Tattini *et al*. 2004, Bidel *et al*. 2007, Solovchenko and Merzlyak 2008, Guidi *et al*. 2011, Klem *et al*. 2015). Earlier studies focused mainly on the UV-shielding (or UV-screening) function of these secondary metabolites (particularly flavonoids and phenolic acids) accumulated in leaf epidermis because of their strong absorption in the UV-B and UV-A regions (Burchard *et al*. 2000, Bilger *et al*. 2001). The epidermal PheCs contributing to UV shielding serve as a first line of UV protection because they are able effectively to attenuate UV radiation before it reaches the sensitive tissues of leaves, thus reducing both direct UV-B damage and ROS formation without substantially reducing PAR, which is important for photosynthesis. Later, it was discovered that PheCs often exhibit antioxidant activity (AOX) and protect plants by scavenging ROS or preventing their production due to chelation of transition metals involved in Fenton's reaction (Hernández *et al*. 2009). The degree of AOX is related primarily to the total number and arrangement of hydrogen-donating hydroxyl groups located on the PheC carbon backbone. Particularly, the presence of catechol group on the flavonoid B-ring (3′,4′-B-dihydroxylated flavonoids) and hydroxylation in position 3 significantly increases AOX of flavonoids. Similarly, the occurrence of catechol group on the aromatic ring (position 3, 4) of hydroxycinnamic acid markedly enhances their ability to scavenge ROS. Also other functional groups and structural features have a positive effect on PheCs AOX, such as 2-3 unsaturated bond or 4-oxo group located on the flavonoid C-ring. In contrast, methoxylation and glycosylation usually decrease AOX. It is also known that the linkage of carboxyl group to aromatic core affects AOX; hydroxycinnamic acids are stronger antioxidants compared to corresponding hydroxybenzoic acid (Rice-Evans *et al*. 1996, Heim *et al*. 2002). It has been reported that PheC antioxidants are accumulated also in chloroplasts (Mondolot *et al*. 2006, Agati *et al*. 2012), where ROS are intensively produced under high irradiance.

The main photoprotective functions of Cars relate to their binding in pigment-protein complexes of both photosystems: *1*) β-carotene serves in particular as an efficient scavenger of $^1O_2$ in the core complexes of both photosystems (Telfer 2002, Cazzaniga *et al*. 2012); *2*) lutein acts as the main quencher of triplet state Chl in light-harvesting complexes (LHCs) (Mozzo *et al*. 2008); and *3*) zeaxanthin and probably also antheraxanthin and lutein mediate ΔpH-dependent heat dissipation of excitation energy in the antenna complexes of PS II, thereby preventing the production of ROS in PS II (Štroch *et al*. 2004b, Horton *et al*. 2008, Jahns and Holzwarth 2012). Under prolonged high irradiance, the pool of xanthophyll cycle pigments (VAZ) increases. Maximum degree of violaxanthin is converted by de-epoxidation, zeaxanthin epoxidation slows, leading to larger, rapidly inducible and more stable heat dissipation (Kurasová *et al*. 2002, Štroch *et al*. 2008a, Demmig-Adams *et al*. 2012). In addition to zeaxanthin bound to LHCs, "free" zeaxanthin within lipid phase of thylakoid membranes serves as a strong antioxidant and membrane stabilizer (Havaux and Tardy 1997, Havaux *et al*. 2007), particularly in mutants with reduced size of LHCs of PS II (LHC II). These include, for example, Chl *b*-less mutants of *Hordeum vulgare* L. (*Clo f2*, Havaux and Tardy 1997, Štroch *et al*. 2004a) and *Arabidopsis thaliana* L. (*Ch1*, Havaux *et al*. 2007, Dall'Osto *et al*. 2010).

Whereas the activation of photoprotective processes mediated by PheCs and Cars after exposure of plants to high PAR and/or UV has been intensively studied for more than two decades (*e.g.,* Edreva 2005, Demmig-Adams and Adams 2006), information on the relaxation of plant photoprotection after lowering irradiance is still rather scarce (Barnes *et al*. 2013). After exposure of plants to higher PAR, UV shielding usually increases for several days before it reaches the optimum for the given conditions, as reported by Agati *et al*. (2011; *ca* 8 d after transfer of *Ligustrum vulgare* L. from 30 to 85 % sunlight), Štroch *et al*. (2008b; *ca*. 6 d after transfer of *H. vulgare* from a greenhouse to full sunlight), and Barnes *et al*. (2013; 5 to 15 d depending on UV conditions, after transferring shade-acclimated *Populus tremuloides* to ambient sunlight). According to our knowledge, the only study on the response of UV shielding after transferring of *P. tremuloides* and *Vicia faba* L. from full sunlight to shade conditions showed the stability of UV screening for at least several days (Barnes *et al*. 2013). It is widely accepted that the acclimation response of the photosynthetic apparatus, including adjustment of the VAZ pool, to both increased and reduced PAR is fully flexible and completed within a couple of days (Demmig-Adams *et al*. 2012). Again, there are fewer studies on the dynamics of acclimation for plants grown under high PAR to shade conditions in comparison to the opposite radiation change. Förster *et al*. (2009) found that in young sun-acclimated avocado leaves, the minimum VAZ pool size was reached *ca*. 6 d after shading (10 % of the full sunlight).

In our previous study, we proved that both stimulation of UV shielding and accumulation of Cars occur within 6 d after transferring WT and *Clo f2* plants from a green-house to outdoor conditions (Štroch *et al*. 2008b). We

found that limited stimulation of UV shielding in *Clo f2* leaves acclimated to full sunlight in comparison to WT plants was compensated by more pronounced accumulation of Cars. Moreover, it was suggested that particularly in *Clo f2* plants, the accumulation of xanthophyll cycle pigments within the lipid phase of thylakoid membrane contributes to resistance to high PAR stress (Štroch *et al*. 2004a).

In the present study, we analyzed changes in the composition of PheCs and Cars in leaves of spring barley (WT and *Clo f2*) during acclimation from high PAR (HI) to low PAR (LI) conditions. We attempted to verify the hypothesis that in both WT and *Clo f2* plants, the amount of PheCs remains constant during one week after transferring plants from HI to LI conditions, whereas the VAZ pool size together with the de-epoxidation state in dark-adapted plants promptly adjust to a level close to that typical for plants grown in LI conditions. Further, different contributions of the xanthophyll cycle pigments and PheCs to the flexible protection of the photosynthetic apparatus against oxidative stress during changing radiation conditions in WT and *Clo f2* plants were elucidated. We suppose that the long-term persistence of PheCs pre-accumulated under HI may have important consequences particularly for plant resistance and cross-tolerance.

Materials and methods

Chemicals: All extraction media and solvents for high-performance liquid chromatography (HPLC grade), phosphoric acid for the acidification of mobile phases (90 % purity) and the Tris buffer (99 %), were obtained from *Sigma-Aldrich* (Höhenkirchen, Germany). PheCs standards were obtained from *Extrasynthése* (Genay, France) and *Sigma-Aldrich*, and the purity of all standards was greater than 90 % (see Table 1 Suppl).

Plants and growth conditions: WT spring barley (*Hordeum vulgare* L. cv. Bonus) and its chlorophyll *b*-less mutant *Chlorina f2 (Clo f2*) were cultivated from seeds for 8 d in a growth chamber (*BioLine HB1014, Heraeus Vötch - Industrietechnik*, Hanau, Germany) under high irradiance (HI; 1 000 μmol m^{-2} s^{-1}; 16 h d^{-1}). After this time period, plants were transferred to the low-irradiance conditions (LI; 50 μmol m^{-2} s^{-1}; 16 h d^{-1}) where they were cultivated for another 9 d. The illumination modules of growth chambers were equipped with 100 W krypton bulbs (100W/E27, *General Electric,* Fairfield, USA) and halogen lamps (*POWERSTAR HQI-BT 400 W/D Daylight, Osram,* Munich*,* Germany). Residual UV from artificial light sources was excluded using a *UV226* filter (*LEE filters*, Hampshire, UK). Irradiance near the central part of barley primary leaves was measured using a quantum sensor (*LI-190SA*, *LI-COR Environmental*, Lincoln, USA). Plants were cultivated in 4 500 cm^3 pots (100 seeds per plot) in the mixture (1:1, v/v) of substrate for house plants and gardening substrate (*Agro CS*, Česká Skalice, Czech Republic) without additional fertilizers, and they were regularly irrigated. Throughout the experiment, temperature and humidity were controlled at 20 °C and 65 %, respectively. Plants were sampled after 8 d of growth in HI conditions (HI 8) and then after 1, 3, 6, and 9 d of acclimation to LI conditions (LI 1, LI 3, LI 6, and LI 9).

Epidermal UV-shielding assessment: The epidermal UV-shielding capacity of PheCs in the epidermal layers of spring barley leaves was measured using a *Dualex* instrument (*FORCE-A*, Orsay, France). Each leaf was measured in the central area (approximately 3.5 cm from the tip of the leaf). This method is based on the determination of UV-A excited (375 nm) chlorophyll fluorescence attenuation caused by accumulation of UV-A absorbing PheCs, in particular of flavonoids, in the epidermal layer. Assessment of UV-shielding capacity is based on a comparison between chlorophyll fluorescence excited in the red (655 nm) and UV-A regions (Bilger *et al.* 2001, Pfündel *et al* 2007).

Analysis of soluble phenolic compounds: Samples for UV-VIS absorption analysis were prepared by extraction of 100 mg fresh mass of leaf central segments. Leaf material was homogenized in a grinding bowl in 3 cm^3 of 40 % (v/v) methanol and then ultrasonicated (5 min) and centrifuged (3 461 *g* for 5 min; *EBA 20*, *Hettich Zentrifugen*, Kirchlengern, Germany). After centrifugation, 1 cm^3 of a supernatant was diluted to the total volume of 5 cm^3 and measured in a quartz cuvette on a double beam UV-VIS absorption spectrophotometer (*UV550, Unicam*, Cambridge, UK) in the spectral range of 200 to 750 nm.

HPLC-DAD analysis of soluble phenolic compounds: For HPLC-DAD analysis of the PheC profile, 1 cm^3 of undiluted supernatant from the aforementioned sample preparation procedure was used. The extract was filtered through a 0.2 μm *Teflon* filter before analysis. The HPLC-DAD separation method was performed according to Kolb and Pfündel (2005) with some modifications. For additional standardization, 24 standards of PheCs, mainly phenolic acids, flavonoids, stilbenes, and their derivatives, dissolved in methanol were used (see Table 1 Suppl.). All samples (including standard solutions) were analyzed using a *TSP Analytical HPLC-DAD* system (*TSP Analytical*, Alexandria, USA) equipped with a *LiChrosphere RP-18*, 5 μm (4.6 × 250 mm) chromatographic column (*Merck*, Darmstadt, Germany). A gradient of acidified water (H_2O - 850 cm^3; H_3PO_4 - 0.1 cm^3) and acidified methanol solution (methanol - 765 cm^3; H_2O - 85 cm^3; H_3PO_4 - 0.1 cm^3) was used for separation. The elution program was set according to Kolb and Pfündel (2005). Flow of the mobile phase was set to 1 cm^3 min^{-1}

throughout the analysis. Chromatograms were detected at 220, 314, and 440 nm, and UV-VIS spectra were recorded from 200 to 500 nm. The signal recorded at 314 nm was used for relative quantification of spring barley leaf PheCs while the other two signals were used mainly for detection of impurities in samples, such as photosynthetic pigments.

UV-VIS absorption analysis and HPLC-DAD analysis of photosynthetic pigments: Central segments of spring barley primary leaves (100 mg) were used. Before the analysis, the leaf exposed area was determined to which the content of each pigment was then related. Leaves were sampled for the analysis after at least 2 h of dark adaptation. Photosynthetic pigments were extracted using 100 % acetone and then centrifuged at 3 461 *g* for 3 min (*EBA 20*). The supernatant was diluted to a total aceton concentration of 80 % (v/v) and filtered (0.2 μm). Photosynthetic pigment quantity was estimated spectrophotometrically (*UV/VIS 550, Unicam*, Cambridge, UK) according to Lichtenthaler (1987) and also after HPLC (*TSP Analytical*, Alexandria, USA) separation of individual pigments, which is described in Kurasová *et al*. (2003). Absorption spectra were measured between 190 and 750 nm. Chromatograms for quantification were detected at 440 nm. Peak areas of individual pigments were divided by appropriate conversion factors according to Färber and Jahns (1998). De-epoxidation state of xanthophyll cycle pigments (DEPS) was calculated as the sum of antheraxanthin and zeaxanthin content divided by the sum of violaxanthin, antheraxanthin and zeaxanthin.

Statistical analysis: The statistical analysis of biochemical and physiological parameters during acclimation from HI to LI was performed separately for WT and *Clo f2* plants using one-way *ANOVA* followed by Tukey's post-hoc test. Mutual comparison of WT and *Clo f2* samples in particular days of acclimation was carried out using a *t*-test. Sample sets which were assigned as significantly different were labeled using identical symbols (*, +, x, #, a, b, c, *etc*.). The number of identical symbols reflects observed *p*-value intervals (*e.g.,* * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001). Absence of above mentioned symbols indicates non-significant differences among sample sets.

Results and discussion

The epidermal UV shielding did not decrease during acclimation from HI to LI conditions. This leaf optical parameter remained constant in *Clo f2* samples and was even slightly increased in WT samples, mainly during the final phase (LI 9) of acclimation (Fig. 1). The primary leaves of WT plants exhibited significantly higher values ($P < 0.01$ for HI 8 and LI 1; $P < 0.001$ for LI 3, LI 6, and LI 9) of epidermal UV shielding compared to corresponding *Clo f2* samples (25 - 42 %) throughout the experiment, and the difference between WT and *Clo f2* plants had an increasing tendency during growth. We can therefore conclude that those PheCs accumulated under HI, which are involved in UV shielding, were rather stable once synthesized in the epidermal layers of barley primary leaves and that the transition to LI conditions was unable to negatively regulate UV shielding in fully developed leaves.

In our previous experiments, we proved that the UV shielding of barley leaves is positively influenced by high PAR in field conditions (Štroch *et al*. 2008b, Klem *et al*. 2015), particularly in younger leaves (Klem *et al*. 2012). We had observed a significant positive effect of PAR on barley leaf UV shielding during 2 weeks of cultivation in growth chambers, as well. Plants cultivated under both HI and LI conditions retained rather constant levels of UV shielding during their growth (see Fig. 1 Suppl.), thus supporting the aforementioned interpretation of stable UV shielding during acclimation to LI conditions. The present findings are therefore in accordance with the results reported by Barnes *et al*. (2008, 2013) and support the hypothesis that leaves exposed to higher PAR are well acclimated to resist excessive radiation (PAR and/or UV) for at least 1 week after reduction of PAR.

The UV-VIS absorption analysis of PheC leaf extracts

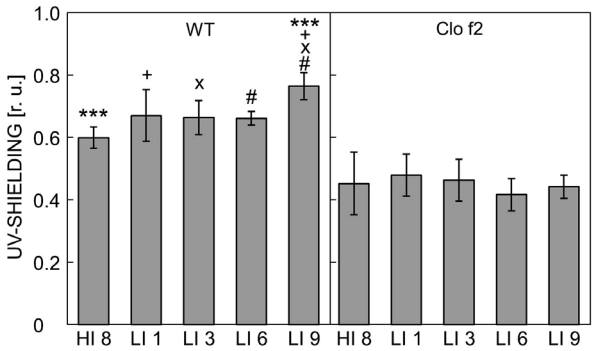

Fig. 1. Epidermal UV shielding of primary leaves of WT and *Clo f2* plants cultivated 8 d under high irradiance (HI 8) and then transferred to low irradiance and sampled after 1, 3, 6, and 9 d (LI 1, LI 3, LI 6, and LI 9). Means ± SDs, $n = 5 - 6$.

revealed two main absorption bands: a narrow band in the UV-B region with a maximum at 270 nm (A_{270}) and a broader absorption band in the UV-A region with a maximum at 335 nm (A_{335}) (Fig. 2). Based on the knowledge of absorption maxima and the shape of absorption bands of individual PheC classes, we concluded that the main compounds of barley primary leaf extracts are mono-B hydroxylated flavones. Comparison of a representative leaf extract spectrum with our PheC spectral library revealed evident similarity

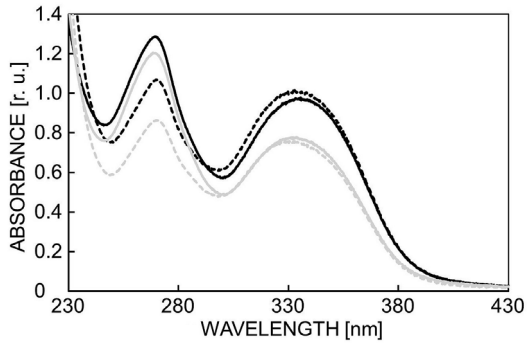

Fig. 2. Alteration of absorption spectra of PheC extracts before and after acclimation of plants from HI to LI conditions. *Black lines* - WT barley, *gray lines* - *Clo f2*; *solid lines* - plants grown 8 d under HI, *dashed lines* - plants after 9 d of acclimation to LI (means of six plants for each treatment).

to the spectrum of the saponarin standard (saponarin was further confirmed by HPLC-DAD analysis as the main PheC of leaf extracts, see below).

During acclimation of plants from HI to LI conditions, we observed a change in A_{270}/A_{335} ratio, while A_{335} remained constant, A_{270} significantly decreased in both WT and *Clo f2* plants and this trend was more pronounced in *Clo f2* plants (Fig. 3). It seems that HI to LI acclimation selectively down-regulated accumulation of compounds strongly absorbing in UV-B close to 270 nm but less or not in UV-A close to 335 nm. This effect could scarcely be explained by the change in the accumulation of detected flavonoids and/or hydroxycinnamic acids (Fig. 4) inasmuch as barley flavonoids (such as saponarin) have almost equal absorbance at the UV-A and UV-B absorption maxima (or lower in UV-B) and phenolic acids exhibit a single broad absorption band of different shape covering both the UV-A and UV-B regions (see Fig. 5*A*,*D*,*G* or results published by Kolb and Pfündel 2005). The compound (or compounds) responsible for the decrease in A_{270} have not yet been identified. The unchanged value of A_{335} indicates that the major flavonoid constituents did not respond to lowered irradiance, although the small hypsochromic shift of the UV-A absorption band may imply a specific alteration of the leaf PheC profile.

PheC leaf extracts prepared from WT plants displayed higher A_{335} compared to those from *Clo f2* plants at all stages of HI to LI acclimation. Taken together with the results of epidermal UV shielding, our data indicate an interconnection of the whole leaf soluble flavonoid content (or at least of the main flavonoid constituents in

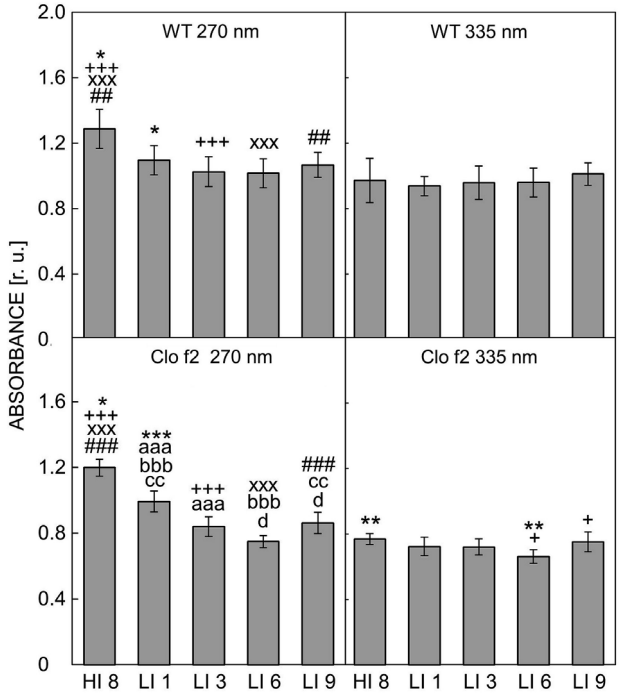

Fig. 3. Temporal changes in absorbance of PheC leaf extracts at 270 nm and 335 nm during cultivation of WT and *Clo f2* plants in high irradiance for 8 d (HI 8) and 1, 3, 6, and 9 d after transferring plants to low irradiance (LI 1, LI 3, LI 6, and LI 9). Means ± SDs, $n = 6$.

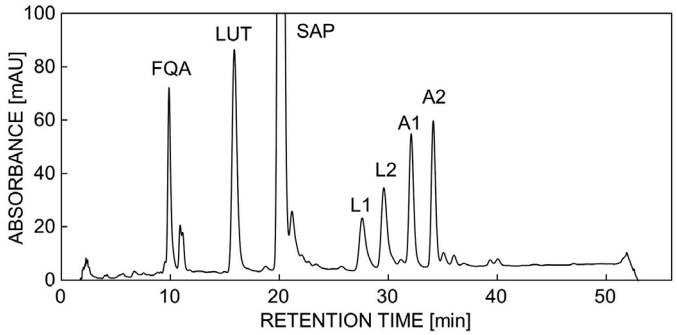

Fig. 4. A representative chromatogram of PheC leaf extract detected at 314 nm. Major peaks belonging to PheCs are depicted (retention times and main absorption maxima are shown in brackets; sh - position of absorption band shoulder). FQA - feruloylquinic acid (10.5 min; 295 sh, 325 nm), LUT - lutonarin (15.5 min; 258 sh, 269, 349 nm), SAP - saponarin (21.0 min; 270 sh, 335 nm), L1 - luteolin derivative 1 (27.5 min; 250 sh, 271, 341 nm), L2 - luteolin derivative 2 (29.5 min; 257 sh, 270, 338 nm), A1 - apigenin derivative 1 (32.0 min; 271, 336 nm), A2 - apigenin derivative 2 (34.0 min; 271, 333 nm)

extracts) and epidermal flavonoid UV-shielding capacity (in the UV-A region) of barley primary leaves. These results are in accordance with those reported by Kolb and Pfündel (2005), who stated a correlation between fluorometrically detected UV-A (360 nm) and UV-B (314 nm) leaf absorbance and the content of luteolin derivatives in leaves of spring barley. Similarly, Barnes *et al*. (2013) observed an association between leaf UV transmittance (375 nm) and alterations in the concentration of total UV-absorbing compounds in *P. tremuloides* and *V. faba* leaf extracts under various irradiances. Nevertheless, Liakoura *et al*. (2003) concluded that epidermal transmittance may not necessarily reflect qualitative and quantitative changes of total leaf PheCs and thus did not recommend assessing the UV-protective potential of leaves based solely on determination of UV-B absorption of methanolic leaf extracts, and particularly not without knowing the leaf architecture, protective pigment localization, and other species-specific parameters.

We can conclude that A_{375} is interconnected to the UV-A shielding capacity of spring barley primary leaves but does not reflect changes in specific UV-absorbing compounds (particularly those responsible for changes in A_{270}, which can affect UV-B attenuation of leaves). Clearly, the determination of epidermal flavonoids involved in UV shielding using *Dualex* cannot reflect the kinetics of unknown UV-absorbing compounds (if they do not absorb UV-A), even though they could participate in total leaf UV-shielding capacity and UV-B protection.

Prior to the analysis of soluble PheCs in leaf extracts, the HPLC separation method described in Kolb and Pfündel (2005) was modified and standardized using 24 PheC standards belonging to the hydroxybenzoic acid, hydroxycinnamic acid, flavonoid, and stilbene PheC subclasses. Calibration equations were established for quantifying these PheCs and a database of their spectra (200 - 750 nm; data not shown) and retention times (Rts) applicable for the identification was also created (see Table 1 Suppl.). HPLC analysis of leaf extracts revealed the occurrence of more than 25 UV-absorbing compounds, 14 of which were classified as PheCs on the basis of their spectral properties (Fig. 4). Compounds eluted up to 15 min were mainly hydroxycinnamic acid derivatives (their spectra were similar to the ferulic, caffeic, and chlorogenic acid standards). The major peak among these compounds came from feruloylquinic acid (Rt = 10.0 min; absorption spectrum Fig. 5*A*), identified by mass spectrometry in Klem *et al.* (2015). Starting at the 15th minute of separation, several di-glycosylated and mono-glycosylated flavonoids were eluted. The first flavonoid was tentatively identified as lutonarin (a di-glycosylated derivative of luteolin) due to the similarity of its absorption spectrum (Fig. 5*D*) to that of homoorientin (a mono-glycosylated derivative of luteolin) and its shorter Rt (15.5 min) compared to homoorientin (Rt = 24.5 min). The shorter Rt is a consequence of additional glucose in the lutonarin molecule. The dominant PheC of leaf extracts was identified to be saponarin, as it exhibited the same Rt (21 min) and absorption spectrum (Fig. 5*G*) as did the saponarin standard. Subsequently eluted most abundant flavonoids were tentatively identified as luteolin and apigenin derivatives based on their absorption properties (Fig. 4). The PheC chromatographic profile is in accordance with the results published by Kolb and Pfündel (2005) and with the identification of individual PheCs analysed using mass spectrometry in our previous experiments with field-grown barley (Klem *et al*. 2015).

Individual PheCs responded differently to the transition from HI to LI conditions. The amount of the main hydroxycinnamic acid derivatives was significantly decreasing during LI acclimation (Fig. 5*B*,*C*). Feruloylquinic acid content in LI 9 was lowered to approximately 5 % of the amount observed in HI 8. It is known that the content of hydroxycinnamic acid derivatives decreases also during aging (Liu *et al*. 1995). Nevertheless, we found that in barley leaves grown under continuous HI, the reduction in feruloylquinic acid was mitigated as compared to that in LI-grown plants of the same age (unpublished data). This indicates that the strong decrease in feruloylquinic acid, as reported here, could be at least in part a response to LI acclimation. It seems that these compounds may be important during

early stages of development, when their quantities in leaf extracts are considerably higher and can be of biological relevance, whereas in later stages, other PheCs (mainly flavonoids) may substitute for their function. The possible photoprotective role of hydroxycinnamic acids in the early-developing photosynthetic apparatus is also supported by the facts that these compounds often exhibit strong antioxidant properties (Rice-Evans *et al*. 1996) whereas their synthesis and localization is closely associated with a chloroplast in young leaves (Mondolot *et al*. 2006).

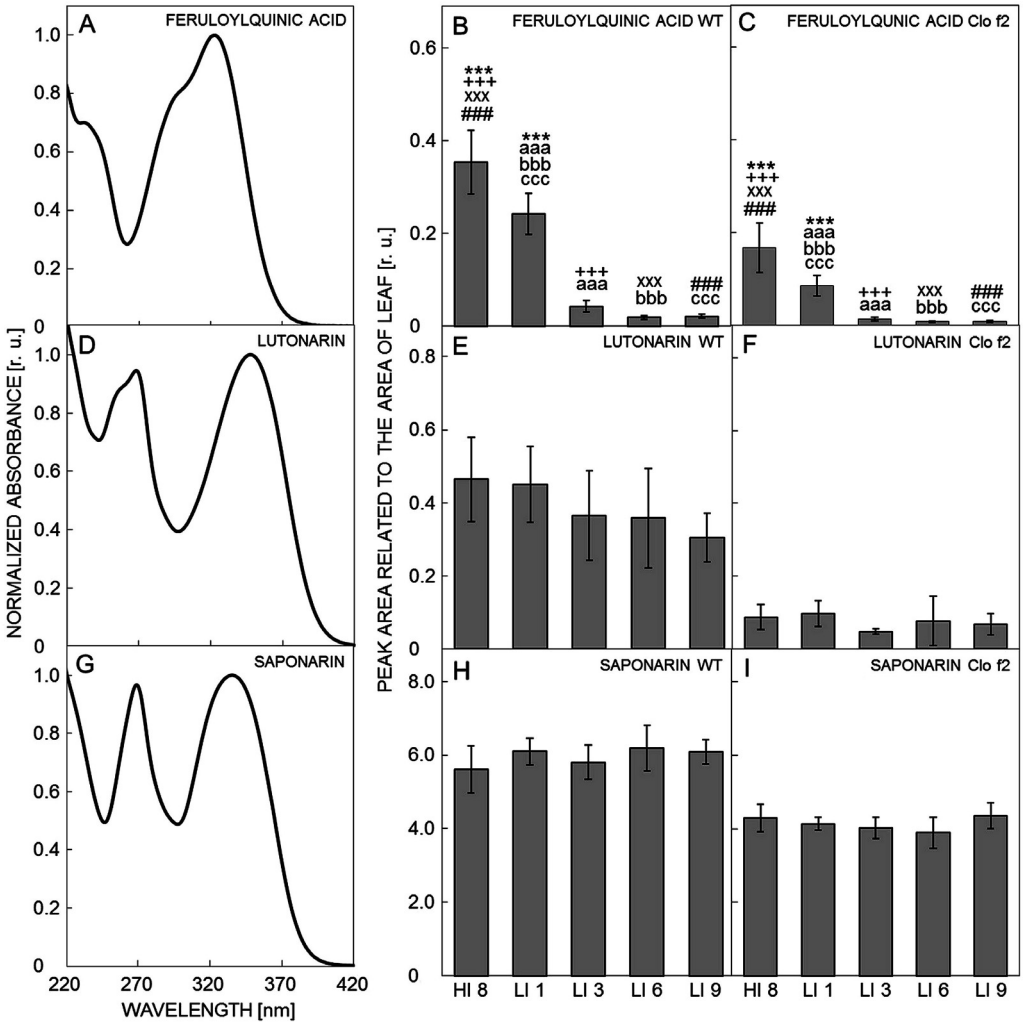

Fig. 5. Changes in the relative quantity of three representative soluble PheCs, feruloylquinic acid (*B* and *C*), lutonarin (*E* and *F*), and saponarin (*H* and *I*), detected in leaf extracts obtained from WT (*B,E,H*) and *Clo f2 (C,F,I*) plants cultivated 8 d under high irradiance (HI 8) and then transferred to low irradiance and sampled after 1, 3, 6, and 9 d (LI 1, LI 3, LI 6, and LI 9). Means ± SDs, $n = 6$. UV-VIS absorption spectra of these compounds are also shown (*A,D,G*).

The main PheC of barley primary leaves, saponarin, was rather unresponsive to the change of irradiance (Fig. 5*H,I*). Similarly, the content of other two apigenin derivatives detected in the samples remained constant or increased slightly (mainly in WT plants) during the transition from HI to LI conditions (see Fig. 2 Suppl.). Kaspar *et al*. (2010) reported that saponarin is accumulated particularly in epidermal layers. Taken together with our results, it seems that constant UV shielding is mainly a consequence of saponarin stability in LI conditions. This also explains the rather constant A_{335} absorbance of leaf extracts after transfer from HI to LI revealed by UV-VIS absorption analysis (Fig. 3). The long persistence of HI PAR pre-accumulated PheCs which can be located in epidermis (such as in the case of saponarin) suggests that they may be involved in regulating other subsequently acting environmental signal responses (such as UV resistance locus 8-mediated UV-B or UV-A responses) and possibly could improve the plant resistance to acute UV-B stress.

In contrast, lutonarin, which is one of the most abundant luteolin derivatives in barley leaf extract, decreased by 34 % during WT plant acclimation to LI conditions (Fig. 5*E*). In *Clo f2* samples, its content did

not reveal a significant reduction (Fig. 5*F*) and it was markedly lower than in WT plants both in HI-acclimated plants (by 81 %) and during acclimation to LI (by 77 to 86 %). *Ortho*-B-dihydroxylated flavonoids, such as lutonarin (and other luteolin derivatives) are well-known plant antioxidants, which may be involved in the reduction of oxidative pressure in chloroplast (Agati *et al*. 2007, 2012). There is a broad evidence that the accumulation of *o*-B-dihydroxylated flavonoids is often positively affected by higher PAR, whereas B-monohydroxylated flavonoids are rather unresponsive (Tattini *et al*. 2004, Agati *et al*. 2011, Klem *et al*. 2015). Our results show that the accumulation of lutonarin is gradually reduced in WT plants during acclimation to LI, whereas flavonoids involved in UV shielding (such as saponarin) as well as other two luteolin derivatives (see Fig. 3 Suppl.) were retained for several days in barley primary leaves. This sensitive PAR dependent regulation of lutonarin content suggests more specific photoprotective function of lutonarin amongst B-dihydoxylated flavonoids of barley. The difference in lutonarin content as well as in the content of other two luteolin derivatives

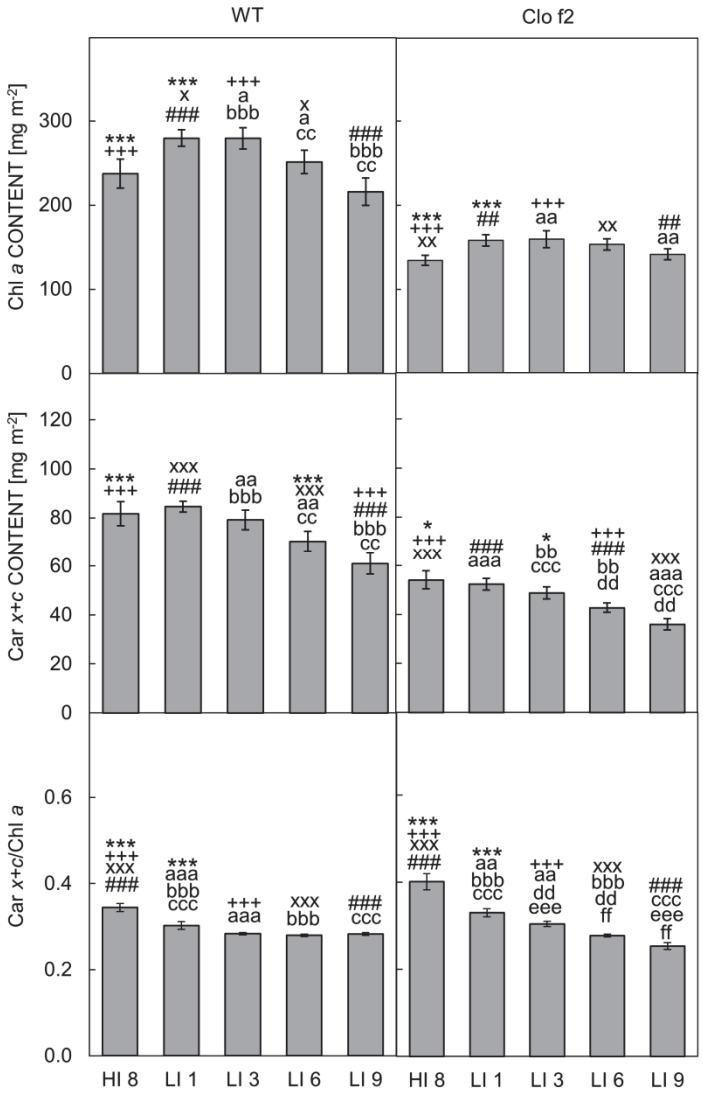

Fig. 6. Spectrophotometrically estimated Chl *a*, Car *x*+*c*, and their ratio (Car *x*+*c*/Chl *a*) in WT and *Clo f2* plants cultivated 8 d under high irradiance (HI 8) and then transferred to low irradiance and sampled after 1, 3, 6, and 9 d (LI 1, LI 3, LI 6, and LI 9). Means ± SDs, $n = 6$.

between WT and *Clo f2* plants may be a consequence of the higher demands for antioxidants in WT chloroplasts under HI conditions compared to those in *Clo f2* plants.

WT and *Clo f2* plants showed the same trend of changes in Chl *a* and total Car content after the reduction of irradiance (Fig. 6). An initial increase in Chl *a* content observed already 1 d after transferring plants to LI conditions (by 18 % for both WT and *Clo f2* plants) was followed by a stable phase until the third day of LI acclimation and a subsequent decrease, which was more

pronounced for WT plants (by 23 % for WT plants and 11 % for *Clo f2* plants). The similar trend as for Chl *a* in WT was observed also for Chl *b* and the content of Chl *a*+*b* (data not shown). Car *x*+*c* content gradually decreased during LI acclimation, more pronouncedly in *Clo f2* plants (by 25 % in WT and 34 % in *Clo f2* plants). A decline in the content of both Chl *a* and Car *x*+*c* was also found in WT plants grown under constant HI (data not shown). This could suggest that the decrease in Car *x*+*c* content in plants transferred from HI to LI conditions reflected not only reduced demands for photoprotection but also changes associated with leaf aging. Primary barley leaves 2 weeks old and older have previously been characterized by a loss of chlorophylls due to an early phase of senescence (Humbeck and Krupinska 2003). The decrease in pigments could also be due to a reduced leaf thickness, but this is not probable in our case as we observed an unchanged ratio of fresh mass to leaf area (Fig. 4 Suppl.).

Different degrees of decrease in both Chl *a* and Car *x*+*c* in WT and *Clo f2* plants led to a different trend of the Car *x*+*c*/Chl *a* ratio (Fig. 6); whereas WT barley leaves reached the lowest value on the third day of LI acclimation, in *Clo f2* plants, it gradually decreased until the end of the experiment. The entire reduction of the Car *x*+*c*/Chl *a* ratio during acclimation to LI was 18 % in WT plants and 37 % in *Clo f2* plants.

A characteristic response to LI is the reduction of VAZ pool size (Demmig-Adams 1998, Kurasová *et al*. 2002), which was observed already 1 d after transferring plants to LI conditions (Fig. 6). HPLC analysis of individual Cars (not shown) revealed that mainly a reduction in the VAZ pool contributed to the reduction of total Cars. The VAZ pool decreased during LI acclimation by 46 % for WT plants and 51 % for *Clo f2* plants. The extent of the decrease in the VAZ pool during acclimation to LI was very similar to the differences between LI- and HI-grown WT and *Clo f2* plants (Štroch *et al*. 2004a). During 9 d of acclimation to LI, therefore, a VAZ pool size typical for LI-grown barley was reached in both WT and *Clo f2* plants (Štroch *et al*. 2004a). The amount of DEPS diminished considerably during LI treatment (Fig. 7). After 1 d under LI conditions, DEPS decreased by about 50 % for both WT and *Clo f2*. Whereas DEPS in HI-acclimated *Clo f2* plants was significantly higher than in WT plants, a similar residual DEPS was found in WT and *Clo f2* plants after 3 and more days of acclimation to LI.

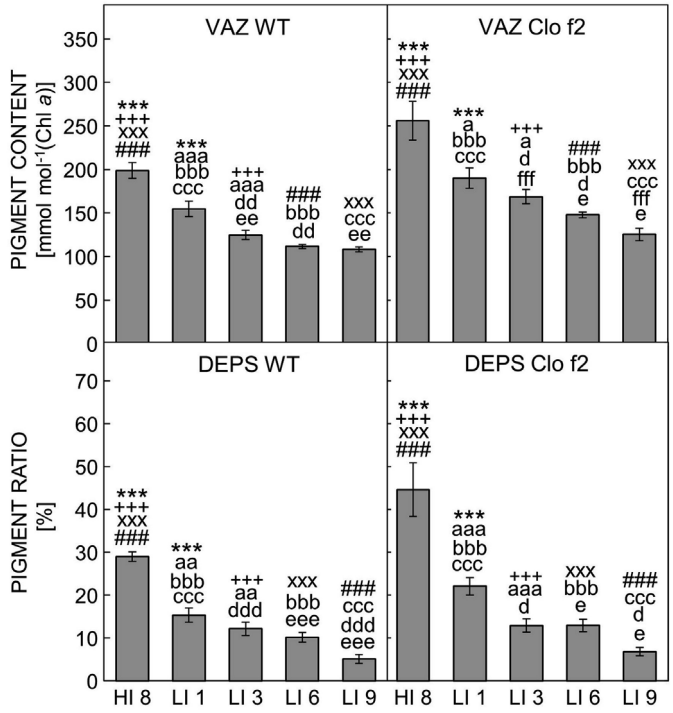

Fig. 7. Total amount of VAZ and their DEPS in WT and *Clo f2* plants during acclimation from high to low PAR conditions. Plants were cultivated 8 d under high irradiance (HI 8) and then transferred to low irradiance and sampled after 1, 3, 6, and 9 d (LI 1, LI 3, LI 6, and LI 9). Means ± SDs, $n = 6$.

We can conclude that after a considerable decrease in irradiance, barley plants flexibly and rapidly (in few days) adjust their composition of photosynthetic pigments. Especially noteworthy is the flexibility of the content of the xanthophyll cycle pigments and their de-epoxidation state in *Clo f2* plants. Among individual xanthophylls, only the relative amount of the VAZ pool was significantly higher in *Clo f2* leaves acclimated to HI as compared to the WT counterpart. Significantly higher VAZ pool size and DEPS in HI-acclimated *Clo f2* plants are probably related to a greater amount of de-epoxidized xanthophylls localized in the lipid phase of thylakoid

membrane than in WT (Štroch *et al*. 2004a). This assumption is supported by the higher values of the ratio of VAZ to lutein in *Clo f2* plants in comparison to WT plants (Fig. 5 Suppl.). Free xanthophylls assure efficient photoprotection of the photosynthetic apparatus of *Clo f2* plants through their antioxidant and screening functions (Havaux and Tardy 1997, Štroch *et al*. 2004a). They could capture excitation energy, thereby prevent the excitation of chlorophyll molecules. Thus, in addition to the well-known flexibility of LHC II size and efficiency of thermal energy dissipation within LHC II in response to changes of irradiance (Demmig-Adams 1998), our results for *Clo f2* response to LI confirm also the flexibility of the content of free xanthophyll cycle pigments with antioxidative function.

In agreement with our previous study (Štroch *et al*. 2008b), we found a different contribution of VAZ pigments and PheCs to photoprotection in WT and *Clo f2* plants grown under HI. The higher degree of UV-shielding induction in WT plants and a greater enhancement of the VAZ pool in *Clo f2* plants were confirmed. The minor importance of PheCs-mediated photoprotection in HI-grown *Clo f2* was supported also by the considerably lower content of lutonarin, a compound with an antioxidative function (Fig. 5).

Conclusions

Our study extended the rather scarce information about the response of PheCs and Cars after a reduction in irradiance. Our original hypothesis was confirmed only in part. We documented a stable UV-shielding efficiency as well as the content of HI-accumulated flavonoids participating in epidermal UV shielding (including the most abundant PheC saponarin). In addition to the expected flexible adjustments of VAZ pool size and de-epoxidation state, however, the content of several specific PheCs with presumed antioxidative functions and chloroplast localizations, such as lutonarin and feruloylquinic acid, tended to decrease during acclimation to LI. Thus, adjustments of both PheCs and pigments with antioxidant activity can contribute to flexible regulation of chloroplast antioxidative capacity after elimination of HI-induced oxidative stress.

We further showed that in HI-grown *Clo f2* plants, accumulation of PheCs with relevant antioxidative activity, particularly lutonarin, was affected to a considerably higher degree in comparison to that of UV-shielding compounds. This indicates that free zeaxanthin within the lipid phase of thylakoid membranes can compensate for the insufficient efficiency of antioxidative protection mediated by PheCs in *Clo f2* chloroplasts acclimated to HI. As in *Clo f2*, the amount of xanthophyll cycle pigments bound in pigment-protein complexes was diminished, and the considerably larger pool in the lipid phase of thylakoid membranes was supported by the significantly enhanced amount of xanthophyll cycle pigments in *Clo f2* acclimated to HI as compared to WT plants acclimated to HI. Reallocation of xanthophyll cycle pigments can thus represent what might be termed a "low-cost" way to reduce photooxidative stress, particularly in *Clo f2*. The results presented herein indicate that both PheCs and VAZ pigments can contribute to flexible modulation of chloroplast antioxidative capacity and that those factors regulating the mutual complementarity of individual antioxidants remain to be elucidated.

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