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Response of the photosynthetic apparatus to UV-A and red light in the phytochrome B-deficient *Arabidopsis thaliana* L. *hy3* mutant[#]

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Abstract

The effect of UV-A radiation (365 nm) and the protective effect of preillumination with red light (RL, 664 nm, 10 min) or with a combination of red and far-red light (FRL, 727 nm, 10 min) on the activity of the PSII as well as the expression levels of selected genes, especially those encoding chloroplast proteins (sAPX, tAPX, CAB1, and D1), were studied in leaves of the 26-d-old *hy3* mutant of *Arabidopsis thaliana*, which is deficient in the phytochrome B apoprotein. The effects were compared with corresponding effects observed in the *hy2* mutant of *A. thaliana*, which is deficient in the phytochrome chromophore. Illumination with UV-A decreased the photosynthetic pigment content, the maximum photochemical quantum yield of PSII (F_v/F_m), and the effective quantum yield of PSII (Φ_{PSII}). The reduction of the F_v/F_m ratio and Φ_{PSII} was more pronounced in the mutants as compared to wild-type plants (WT). The preillumination of the leaves with RL caused a significant reduction in the inhibitory effect of UV-radiation on the PSII activity in the WT plants, but it caused only a small decrease in the *hy3* mutant. The preillumination of leaves with RL and FRL combination compensated the protective effect of RL on the UV-induced decrease of the fluorescence parameters in the WT. Such reversibility is typical for involvement of red/far-red reversible phytochromes at low intensity light. The results suggest an important role of red/far-red reversible phytochromes (phytochrome B) in the resistance of PSII to UV-A radiation caused by changes in contents of either carotenoids or other UV-absorbing pigments probably through biosynthesis of these pigments. The data also demonstrated that phytochrome B and other phytochromes can affect the PSII stress resistance by the fast regulation of the expression of genes encoding antioxidant enzymes and transcription factors at the step of gene transcription.

Additional key words: *Arabidopsis thaliana*; chlorophyll *a* fluorescence; photosystem II; phytochrome system; stress resistance; transcription; ultraviolet.

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Abbreviations: APX1 – cytosolic ascorbate peroxidase 1; CAB1 – Chl *a*/*b*-binding protein; Chl – chlorophyll; CHS – chalcone synthase; F_v/F_m – maximum photochemical quantum yield of PSII; PA – photosynthetic apparatus; Phy – phytochrome; PIF – phytochrome interacting factor; qPCR – real-time quantitative polymerase chain reaction; RL – red light; ROS – reactive oxygen species; sAPX – stromal ascorbate peroxidase; tAPX – thylakoid ascorbate peroxidase; UAPs – UV-absorbing acidic methanol extractable pigments; WT – wild type; Φ_{PSII} – actual photochemical efficiency of PSII.
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Introduction

High doses of UV-A or UV-B often lead to the damage of the photosynthetic apparatus (PA). Plastoquinones (Q_A, Q_B), the D1, D2 proteins, and the enzyme Rubisco are among the primary targets of UV radiation (Strid *et al*. 1994, Babu *et al*. 1999, Asada 2006). The Mn ions in the PSII Mn_4CaO_5 cluster are also vulnerable to UV irradiation (Szilárd *et al*. 2007, Najafpour *et al*. 2013). However, the UV light-induced damage can also depend on the additional interaction of the PA components with light in the visible region. RL of low intensity can partially alleviate the negative effect of UV radiation on plants and their PA (Joshi *et al.* 1991, Lingakumar and Kulandaivelu 1993, Qi *et al*. 2000, 2002; Sicora *et al*. 2003, Kreslavski *et al*. 2013a,b). Previous studies have shown that low-intensity RL pulses activate the phytochrome system, which triggers protective mechanisms against UV radiation (Kreslavski *et al.* 2013a,b). However, many details of this protection by RL acting *via* the phytochrome system have not been fully clarified so far.

The phytochrome system plays an important role in plant growth and PA development. This concept is in agreement with recent studies on phytochrome mutants of *A. thaliana* with deficiencies in different types of phytochromes, which demonstrated that deletion of phytochromes is critical for plant development (Strasser *et al*. 2010, Zhao *et al*. 2013). Normal seedling greening and plant development is impossible if phytochromes are absent (Strasser *et al.* 2010, Zhao *et al*. 2013). The effects of phytochrome deficiency on photosynthetic parameters have been investigated in previous studies, including the impact on PSII activity (Kreslavski *et al*. 2013b) and chlorophyll (Chl) (*a*+*b*) content (Strasser *et al*. 2010, Zhao *et al*. 2013).

Protective effects against UV can be caused by the RL-induced formation of the FR-absorbing physiologically active form of phytochrome (Phy_{FR}) and/or enhancement of phytochrome biosynthesis as a result of RL illumination (Kreslavski *et al*. 2012). It was suggested that protective effects mainly result from decreased Chl degradation and higher stability of PSII, as well as a reduced damage of thylakoid membranes (Joshi *et al.* 1991, Lingakumar and Kulandaivelu 1993). On the other hand, the decreased phytochrome content can reduce the resistance of PA. For example, *hy2* mutants of *A. thaliana* show the decreased content of PhyB and other phytochromes due to reduced biosynthesis of the phytochrome chromophore, phytochromobilin (Parks and Quail 1991). This *hy2* mutant also showed decreased UV-A resistance of PSII, as determined by delayed luminescence emission (DLE) (Kreslavski *et al*. 2013b). It was also shown that the resistance of PA in *A. thaliana* WT increased after preillumination with RL, whereas in the *hy2* mutant the PSII resistance to UV-A did not change upon the same treatment. It was suggested that the PA resistance to UV radiation depends on the ratio of pro- and antioxidant compounds, which can be affected by PhyB and other phytochromes (Kreslavski *et al*. 2013b). However, many questions remain to be answered. In particular, the role of different phytochromes for the UV resistance of PSII has been studied insufficiently so far.

PhyB, one of the key phytochromes in green plants, is involved in the synthesis of photosynthetic pigments and chloroplast development (Zhao *et al*. 2013), as well as in the synthesis of some photosynthetic proteins and stomatal activity (Boccalandro *et al*. 2009). It is also known that the increased PhyB content or its active form can enhance the resistance of the photosynthetic machinery to environmental stress (Thiele *et al*. 1999, Boccalandro *et al*. 2003, Carvalho *et al*. 2011, Kreslavski *et al*. 2015). In particular, transgenic cotton plants, in which PhyB gene of *A. thaliana* was introduced, showed more than a two-fold increase in the photosynthetic rate and more than a four-fold increase in transpiration rate and stomatal conductance (Rao *et al*. 2011). In addition, the increase of PhyB content in transgenic potato plants (Dara-5 and Dara-12), which are superproducers of PhyB, led to enhanced resistance of PA to high irradiance (Thiele *et al*. 1999) and UV-B (Kreslavski *et al*. 2015). The authors suggested that the increased resistance results from the higher Chl content or enhanced stomatal conductance due to superproduction of PhyB, which is the primary RL-sensor in green higher plants playing a dominant role in comparison to other phytochromes.

The *hy3* mutant of *A. thaliana* has an elongated hypocotyl when grown in white light and is paler in color compared to WT (Chory *et al*. 1989). According to the available data, *hy3* is severely defective in the PhyB apoprotein and, hence, in PhyB (Somers *et al*. 1991). Here, we suggested that the *hy3* mutation decreases the resistance of PA to UV-induced oxidative stress directly in the chloroplasts. This study compared different parameters characterizing the activity of PSII (F_v/F_m, Y_{II}), photosynthetic pigments and ultraviolet-absorbing pigments (UAPs) contents of both mutants (*hy2* and *hy3*) and the WT, including the resistance of PSII to UV-A. New data were presented on the effects of UV, RL, and the combined illumination with RL first and subsequently with FRL (RL→FRL) on the level of an expression of the key antioxidant genes and the photochemical activity of PSII in the *hy3* compared to *hy2* mutent and the WT. The aim of this study was to understand the role of PhyB in the regulation of the photochemical activity of PSII under UV-A stress conditions.

Materials and methods

Plant material, growth conditions, and irradiation schemes: The 26-d-old *hy3* and *hy2* mutant plants of *A. thaliana* (ecotype Columbia-0) (from the European Arabidopsis Stock Centre, Nottingham, UK) were used for our study. The *hy3* mutant plants are deficient in the PhyB apoprotein and *hy2* mutant plants are unable to produce the phytochrome chromophore (phytochromobilin), which caused a deficit in all phytochromes simultaneously.

From seed germination all plants were grown in growth chambers with a 12-h photoperiod under white fluorescent lamps [12 h light, irradiance = 110 μmol(quantum) m^{-2} s^{-1} PAR, 23 ± 0.5°C] and 12 h in the dark (21 ± 0.5°C) for 26 d.

After the last dark period, the pots with the plants or upper developed detached leaves from the plants were exposed to different light treatments. After detachment, the leaves were kept on wet paper in the dark for 0.5 h, and then they were irradiated. In this way, well reproducible illumination conditions were provided. UV-A for 2 h or UV-A (2 h) after preliminary RL exposure (λ_{max} = 664 nm, half-width 32 nm, 10 min) or both, preliminary RL (10 min) and then FRL exposure (λ_{max} = 727 nm, half-width 40 nm, 10 min) were used. Another set of samples was not subjected to the light treatments (control). In some experiments, the light treatment was provided with RL or FRL only. The RL or FRL intensity was 10 μmol(quantum) m^{-2} s^{-1} PAR on the surface of the leaves. The ambient temperature was not changed during and after the irradiation. UV-A was obtained by an ultraviolet lamp (*Selecta T8 18W BLB, Camelion,* China) with λ_{max} = 365 nm and a half-width of 24 nm (irradiance = 10 W m^{-2} on the leaf surface). Both RL and FRL were obtained from light-emitting diodes (*TH NEON Ltd.*, Saint-Peterburg, Russia).

The fluorescence parameters (F_v/F_m, Y_{II}), photosynthetic pigment contents, fresh biomass, and the levels of expression of some key genes encoding antioxidant enzymes, photosynthetic proteins, and components of phytochrome signaling were determined. All experimental steps in the dark were performed under a dim green light.

The total amount of all plants at the end of growing period was approximately 50–100 plants. All experiments were repeated 3–8 times (*n*). Previously, we checked how the fluorescence parameters changed in the dark after UV and RL or FRL irradiations. The changes of fluorescence parameters in the dark after the irradiation with RL and FRL were found to be insignificant for all investigated illumination duration up to 2 h.

Photochemical activity of PA was analyzed by measuring the Chl *a* fluorescence with a pulse amplitude modulation (PAM) fluorimeter (*XE-PAM*, *Heinz Walz*, Germany). The characteristic values F_v, F_0, F_m, and the maximum quantum photochemical yield of PSII (the ratio of F_v/F_m), as well as the effective photochemical quantum yield (Φ_{PSII}) of PSII were determined (Maxwell and Johnson 2000). Φ_{PSII} was calculated as $(F_m' - F_t)/F_m'$, where F_m and F_m' are the maximum fluorescence levels under dark and light-adapted conditions, and F_t is the level of fluorescence before a saturation impulse is applied. To measure maximal Chl fluorescence in the light-adapted state, actinic light was switched on for 10 min [190 μmol(quantum) m^{-2} s^{-1} PAR]. Maximal fluorescence (F_m) was measured using saturating pulses [6,000 μmol(quantum) m^{-2} s^{-1} PAR, 800 ms duration].

For each variant (WT, *hy2*, *hy3*), three or four healthy, developed, upper leaves with almost horizontal position of the leaf blade were chosen from three pots for fluorescence measurements. The leaves were detached, kept in the dark for 30 min, then exposed to light treatments, and kept in the dark at least for 20 min until fluorescence measurements were carried out. All irradiated leaves were compared with samples kept in the dark only (control). Here, we alternated the order of investigation for WT, *hy2*, and *hy3* leaves during the experiment in order to reduce errors.

In some experiments, the plants were exposed to light treatments, then the developed upper leaves with almost horizontal position of the leaf blade were detached, kept in the dark for 30 min, and after that used for fluorescence measurements. This experimental series was repeated 6–8 times for each variant (WT, *hy2*, *hy3*).

Pigment contents and dry mass: The content of the photosynthetic pigments Chl *a* and Chl *b*, as well as that of carotenoids (Car) was determined in ethanol extracts from plant leaves, using the absorption coefficients from Lichtenthaler and Wellburn (1987). For both UV treatment and control without UV irradiation and for each variant (WT, *hy2*, *hy3*), 12–16 leaves from different pots were used. The content of UV-absorbing pigments (UAPs) was determined by the method of Mirecki and Teramura (1984). Leaf discs (*n* = 8–10, 0.2 cm in diameter) were cut from leaves and kept for 24 h in acidic methanol with added HCl and water (methanol:water:HCl = 78:20:2) at 4°C. The UAP content was determined from the absorbance at 327 nm by a spectrophotometer (*Genesis 10UV*, *Thermo Spectronic*, USA) and expressed as relative units per 1 g of fresh leaf biomass (FM).

The dry mass (DM) of the leaves was determined after oven drying at 72°C for 24 h.

Chl *a* fluorescence by fluorescence microscope: The effect of high intense UV-irradiation on the Chl *a* content was studied by registration of the Chl *a* fluorescence with a *TI Eclipse* (*Nikon*, Japan) wide-field fluorescence microscope equipped with 10× objective.

Excitation was performed by a mercury lamp *via* a dichroic cube (*425 DCXRU*, *AHF Analysentechnik*, Tübingen, Germany) with an additional bandpass filter for the selection of the excitation range (*ET Bandpass 360/40*,

AHF Analysentechnik, Tübingen, Germany) and an additional emission filter (*BrightLine HC 682/25*, *AHF Analysentechnik*, Tübingen, Germany) in order to ensure that mainly Chl *a* luminescence was monitored. The filter set was used to select the desired UV-A range from the emission of a mercury lamp and to remove the stray light of the laser used for fluorescence excitation.

All images were recorded with an EM-CCD camera (*Andor-Luca*, *Andor Technology*, Belfast, UK). The intensity of the UV-A light during excitation was 5,000 W m^{-2} and the duration of irradiation was 30 s. In the experiments, three detached leaves from different pots and three replicates for each variant (WT, *hy2*, *hy3*) were used for irradiations. The chloroplasts were visually identified in the microscopic images and evaluated selectively. Eight-chloroplast regions per leaf area were selected for evaluation of the intensity decay (Fig. 1) with *ImageJ* software, followed by statistical averaging over three leaves (altogether 24 data sets per sample).

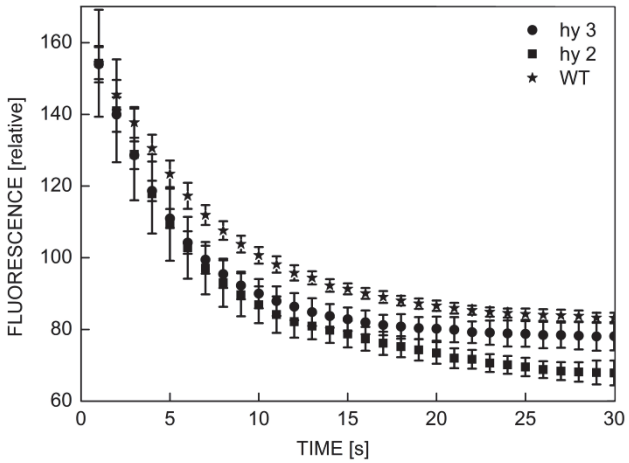

Fig. 1. Dependence of the Chl *a* (682/25 nm) fluorescence emission on the duration of continuous irradiation with UV-A (360/40 nm, I = 5,000 W m^{-2}) in *hy2* and *hy3* mutants compared with the wild type (WT). For each bleaching series, three different leaves of *A. thaliana* WT and mutants grown under white light were bleached and the fluorescence signal was integrated over eight different leaf sections evaluating mainly the fluorescent area of the chloroplasts. The error bars denote the standard deviation of the average, *n* =3.

RNA isolation, cDNA synthesis, and Real-Time quantitative PCR: The leaves of irradiated and nonirradiated plants were collected immediately after irradiation and then frozen at 70°C for 24 h. Three of four leaves from each variant (WT, *hy2*, *hy3*) were taken from three pots and the experiments were carried out five or six times (*n* = 5 or 6). The total RNA was isolated using TRIzol Reagent (*Invitrogen*, USA) according to the manufacturer's instructions. The plant tissue was homogenized in TRI-Reagent and chloroform was added to separate the aqueous and the organic phases. RNA was precipitated from the aqueous phase with isopropanol. The resulting precipitate was washed with 70% ethanol and then dissolved in diethylpyrocarbonate-treated autoclaved distilled water. For the first-strand cDNA synthesis, a reaction kit for reverse transcription (*Synthol*, Russia) was used according to the manufacturer's instructions. A real-time quantitative polymerase chain reaction (qPCR) was done using *iCycler IQ5* (*Bio-Rad*, USA) and reaction mixtures from the qPCRmix-HS SYBR kit (*Evrogen JSC*, Russia). The *UBQ5* (AT3G62250) gene transcript was used as an internal reference (Gutierrez *et al.* 2008). The *Oligo* software was used for primer design.

The primer sequences were:

Sequence 5'-3' Forward primer	Sequence 5'-3' Reverse primer	Accession number
gacgcttcatctcgtcc	ccacaggttgcgttag	AT3G62250 (*UBQ5*)
gataggtctggcttcgaaggt	atgtgggcctcagcgtaatca	AT1G07890 (*APX1*)
gtacacgaaagaaggacctggagca	ttcaaggattacgctgtagcccatg	AT4G08390 (*sAPX*)
ctcccgagggcatagtcattgaaa	agtggacaagaggaccaaaacg	AT1G77490 (*tAPX*)
agaggccgaggacttgctttac	ccaaatggtcagcaaggttctc	AT1G29930 (*CAB1*)
agcgatcctagaccaggtgga	actccaacccttctcctgtcg	AT5G13930 (*CHS*)
gcaagcaagagagaggaaaaagg	cagcattagaaccaccaccacc	AT5G11260 (*HY5*)
gcaacaagcaagagagaggaagaa	ttgtcatcagttttaggccttgtg	AT3G17609 (*HYH*)
caccgggtatggggcattac	tctctagtcgagccattgccaa	AT1G09530 (*PIF3*)
ggcgtgtccaggtgaaggtctg	cgaaagcgaccattgtcaactgc	AT2G18790 (*PhyB*)
ggctttgttctctgcgtttct	gtgcctaactcatccacccc	AT3G09150 (*HY2*)
agtttccgtctgggtatgcg	taaaaagggagccgccgaat	ATCG00020 (*psbA*)

The conditions for qPCR and other details are described in Kreslavski *et al*. (2013b). The results were analyzed as described in Pfaffl (2001). Statistical analysis was carried out using *Microsoft Excel*. All information

about the addressed *A. thaliana* genes was obtained from the database "*The Arabidopsis Information Resource*" (http://www.arabidopsis.org).

Statistical analysis: The tables show the arithmetic means of the obtained values and their standard errors (± SE). The graph was drawn along averaged values and standard deviations (± SD) were denoted graphically. All the experiments were conducted with at least three independent biological replicates. The significance of differences between the variants was described by the *t*-test at the 5% significance level.

Results

The mutant *hy3* had a longer hypocotyl and thinner leaves than that of WT. The *hy2* mutant leaves were also thinner as compared to those of WT (*hy3* by 11 ± 3%, *hy2* by 16 ± 4%), as evaluated from weighing leaf cuttings of 8 mm in diameter. Besides, the *hy2* and *hy3* leaves were paler than those of WT. The ratio of leaf DM to fresh leaf FM was approximately equal in the WT and both mutants (0.087 ± 0.003).

PSII activity and pigment content in leaves without light treatments: Fluorescence parameters, such as the F_v/F_m and Y_{II} were close to each other in *hy3*, *hy2*, and WT (Table 1) with a weak indication that the parameters F_v/F_m and Φ_{PSII} were a bit lower in the mutants than those in WT.

We compared both, the leaves, which were detached after the light treatments, and leaves, which were measured *in vivo*. However, no significant difference in the determined fluorescence parameters was observed between leaves, which were detached before the irradiations and leaves measured *in vivo*. For example, the F_v/F_m ratio was smaller in the detached leaves by about 0.01 compared to the attached native leaves (data not shown).

The content of Chl *a*, Chl *b*, and Car per 1 g(FM) was smaller by 20% in the *hy2* mutant and by 17% in the *hy3* mutant as compared with WT. The content of UAPs was also smaller in the mutants compared with WT (Table 2).

Effect of UV-A and red light preillumination on fluorescence parameters: We compared the effect of UV-A on the PSII activity (F_v/F_m and Y_{II}) in the *hy3* and *hy2* mutants and WT and studied the effect of preillumination with RL on the UV-A-induced inhibition of PSII (Table 1). UV-A irradiation significantly reduced both F_v/F_m and Y_{II}. For example, decreasing F_v/F_m in UV-irradiated WT compared with control WT (control WT is accepted as 100%) was equal to 8.6%, whereas the decreases in *hy3* and *hy2* samples were 10.3 and 10.8%, respectively. Thus, changes in the fluorescence parameters upon UV irradiation in the *hy2* and *hy3* mutants were more pronounced than those in WT. The difference in fluorescence parameters between *hy2* and *hy3* was small. Y_{II} of the PSII was more sensitive to the UV treatment than the F_v/F_m ratio. Also, the correlation between the values of the F_v/F_m ratio and Y_{II} was observed (Table 1).

Table 1. Effect of UV-A (10 W m^{-2}, 2 h) irradiation and RL (R) and FRL (F) as well as their combinations on the fluorescence parameters: F_v/F_m and Φ_{PSII} in detached leaves of 26-d-old *hy2*, *hy3* mutants, and WT *Arabidopsis* plants. The leaves were detached and kept for 0.5 h in dark. Then they were exposed or not exposed (control) to light treatments. Means ± SE of approx. 30 leaves, $n = 6$. The change of F_v/F_m [%] is equal to [(F_v/F_m (control samples) – F_v/F_m (irradiated samples)]/F_v/F_m (control samples) × 100. * – difference of values in percentage between WT and mutants in UV- or RL-UV-treated samples was reliable ($p<0.05$). Note: A difference between parameters for control WT, *hy2*, and *hy*3 and corresponding parameters for light-treated WT, *hy2*, and *hy3*, respectively, was significant ($p<0.05$), excluding a difference between WT in the control and WT in RL-UV-irradiated samples which was insignificant.

Variant of irradiation		Φ_{PSII}	F_v/F_m	Change of F_v/F_m [%]
Control	WT	0.54 ± 0.02	0.815 ± 0.008	0
	hy3	0.51 ± 0.025	0.80 ± 0.007	0
	hy2	0.50 ± 0.03	0.79 ± 0.008	0
UV	WT	0.44 ± 0.03	0.745 ± 0.01	8.6 ± 0.15
	hy3	0.39 ± 0.03	0.718 ± 0.008	10.25 ± 0.2*
	hy2	0.38 ± 0.025	0.705 ± 0.01	10.8 ± 0.2*
R→UV	WT	0.52 ± 0.015	0.79 ± 0.012	2.6 ± 0.05
	hy3	0.42 ± 0.03	0.74 ± 0.011	7.5 ± 0.2*
	hy2	0.40 ± 0.03	0.71 ± 0.01	10.1 ± 0.2*
R→F→UV	WT	0.46 ± 0.02	0.73 ± 0.017	9.2 ± 0.2
	hy3	0.43 ± 0.03	0.73 ± 0.013	8.75 ± 0.2
	hy2	0.39 ± 0.03	0.71 ± 0.01	10.4 ± 0.15*

Table 2. The content of photosynthetic pigments and UAPs in the leaves of *hy3*, *hy2*, and wild type (WT) of 26-d-old *Arabidopsis* plants grown under white light. Car – carotenoids, UAPs – methanol-extractable UV-absorbing pigments. Data for variant RL→UV instead of UV. The data indicate average values with standard errors for three replicates ($n = 3$). * – difference before and after the UV treatment was reliable ($p<0.05$). Note: FM of leaf segment of 1 cm^2 was 17 mg for WT, 15 mg for *hy3*, and 14.1 mg for *hy2*.

Parameter	WT	WT(UV)	*hy2*	*hy2*(UV)	*hy3*	*hy3*(UV)
Chl *a* [mg g^{-1}(FM)]	336 ± 11	320 ± 12	270 ± 9	238 ± 7*	281 ± 12	259 ± 11
Chl *b* [mg g^{-1}(FM)]	152 ± 14	145 ± 11	122 ± 11	108 ± 8	123 ± 6	114 ± 9
Chl (*a*+*b*) [mg g^{-1}(FM)]	488 ± 16	465 ± 15	392 ± 12	346 ± 10*	404 ± 14	373 ± 14
Chl *a*/*b*	2.2	2.2	2.2	2.2	2.3	2.3
Car [mg g^{-1}(FM)]	80.7 ± 3	78.3 ± 3.3	63.9 ± 1.9	56.8 ± 1.7*	64.6 ± 2.2	61.2 ± 2.9
UAPs [rel. units per 100 mg(FM)]	0.32 ± 0.012	0.28 ± 0.01*	0.27 ± 0.01	0.21 ± 0.01*	0.25 ± 0.009	0.20 ± 0.01*
UAPs [rel. units per 100 mg(FM)]	-	0.305 ± 0.01	-	0.20 ± 0.012	-	0.21 ± 0.008

In addition, the reduction of the PSII activity after UV irradiation in WT samples, which had been preilluminated with RL, was less pronounced than without RL preillumination, whereas in the *hy2* samples no significant protective effect of RL was observed. The *hy3* demonstrated a weak protective effect. Moreover, after RL preillumination, the subsequent illumination with FRL led again to a substantial UV-induced reduction of the PSII activity in WT, which was comparable to the plants not RL-treated at all (Table 1). Small RL-FRL reversibility was observed in the *hy3* mutant and there was no significant reversibility in the *hy2* mutant. The RL and FRL treatments alone had no detectable effect on the PSII activity (data not shown).

Effects of UV-A on pigment contents: There was no significant reduction of the photosynthetic pigments in the WT and *hy3* induced by UV irradiation (Table 2). However, the contents of Chl (*a*+*b*) and Car in the *hy2* leaves exposed to UV irradiation decreased as compared to the dark control by approximately 12 and 11%, respectively. The content of UAPs decreased both in the WT and in mutants but the decrease in the mutants was more pronounced than that in WT. Decrease of UAPs was smaller in the WT samples preillluminated with RL, but in the mutants, the effect of preillumination was insignificant.

There are two processes during long-time UV treatment: pigment degradation and biosynthesis. In order to avoid an interference of UV affecting the pigment biosynthesis *de novo* and to compare the Chl bleaching in *hy2* and *hy3*, we used high UV irradiance and very short irradiation time. In that case, mainly the Chl *a* content should be assessed by the Chl *a* fluorescence. The Chl *a* fluorescence decreased quickly and irreversibly under strong UV-A light in all leaf samples (Fig. 1). The reduction appeared faster in the leaves of the *hy3* and *hy2* mutants as compared with WT. The decay of the red Chl *a* fluorescence was fitted according to the exponential decay function:

$$I(t) = I(0)e^{-\frac{t}{t_1}} + Y_0$$

The resulting fit parameters are $Y_0 = 82$, $t_1 = 6.7 \pm 0.08$ s for WT; $Y_0 = 69$, $t_1 = 5.7 \pm 0.16$ s for the *hy2* mutant; and $Y_0 = 78$, $t_1 = 4.8 \pm 0.04$ s for the *hy3* mutant. The differences between WT and *hy2*, as well as between WT and *hy3* were significant.

Effect of RL illumination and UV light on gene expression: First, we compared the transcriptional levels of some genes encoding antioxidant enzymes and photosynthetic proteins without any irradiations (Table 3). The levels of *CAB1* and *psbA* genes were higher in the *hy2* mutant compared with WT. The activity of the *CHS* gene was lower in the *hy3* mutant than that in WT.

The transcript levels of the antioxidant genes, *APX1* and *sAPX*, of the *hy3* mutant plants increased after 10 min of RL irradiation and the subsequent dark period of 1 h (Table 4). The transcriptional activities of a number of genes in the plants illuminated with RL were presented in the Table 4; the activities of the corresponding genes without RL treatment were accepted as 1.

Transcript levels of the antioxidant genes *APX1* and *tAPX* as well as *PhyB* in the WT plants increased under the same conditions (Table 4). The expression levels of other genes showed no significant changes. Compared to the *hy3* and WT, the transcript levels of the genes *sAPX*, *tAPX*, *CHS*, *HYH*, and *HY5* decreased in the *hy2* mutant. Note that effect of RL on transcript levels of some genes (*HYH* and *ASP1*) was partly eliminated by subsequent irradiation with FRL (Table 4). Upon UV irradiation, the transcript levels of the antioxidant genes *APX1* and *HY5* increased strongly approximately from 3- to 6-fold in *hy3*, *hy2*, and WT (Table 5). Transcription of the *CHS* gene increased only significantly in the *hy3* mutant samples. The transcription of other genes either decreased or changed insignificantly.

Note that joint action of UV-A and RL in WT did not lead to significant enhancement of the effect of RL on the transcriptional levels of genes encoding enzymes of antioxidant system, such as *APX1*, *CHS*, *tAPX* or transcription factors.

Discussion

At least three sites or processes in PA are known to be stress-sensitive to irradiation with UV-A: PSII with its oxygen-evolving complex, the ATP generation, and the carbon assimilation processes (Allakhverdiev and Murata 2004, Nishiyama *et al*. 2006, Murata *et al*. 2007, Allakhverdiev *et al*. 2008).

Chl fluorescence is a very useful and sensitive indicator to study changes in the status of PA and photosynthetic activity. Fluorescence has also been used to monitor effects of various stressful factors on PA activity, first of all, on the PSII photochemistry: pollutions (Tuba *et al*. 2010, Shaw *et al*. 2014), specific nutrient deficiency in plants (Aleksandrov *et al*. 2014, Živčák *et al*. 2014b), environmental stress, for example, drought (Živčák *et al.* 2014a), and salt stress (Oukarroum *et al.* 2015) or high light (Breštič *et al*. 2015), and UV-radiation (Ranjbarfordoei *et al*. 2011).

In our work, we analyzed the effects of UV-A on the PSII activity of *hy3* in comparison with WT and *hy2*. UV-A radiation decreased the photochemical activity of PSII in WT and both mutants of *A. thaliana* (Table 1). However, the mutants were more sensitive to the inhibitory action of UV-A. Wherein a difference between the *hy2* and *hy3* was smaller than that between the mutants and WT, which agrees with concept of the key contribution of PhyB to the resistance of PSII to UV-radiation.

Table 3. The transcriptional activity of antioxidant and photosynthetic genes in the 26-d-old *Arabidopsis* leaves of the WT and mutants *hy3* and *hy2*. Activity of WT is normalized to 1. Means ± SE, $n = 5$. *– reliable differences between WT and *hy2* or *hy3*, $p<0.05$.

Gene	Protein	WT	*hy3*	*hy2*
AT1G07890	APX1	1	0.9 ± 0.05	1.45 ± 0.4
AT4G08390	sAPX	1	0.96 ± 0.3	0.75 ± 0.3
AT1G77490	tAPX	1	1.0 ± 0.09	1.6 ± 0.5
AT5G13930	CHS	1	0.3 ± 0.1*	1.1 ± 0.2
AT1G29930	CAB1	1	1.05 ± 0.1	1.7 ± 0.3*
ATCG00020	D1 (*psbA*)	1	1.6 ± 0.15	3.2 ± 1.3*

Phytochromes are suggested to play an important role in the control of photosynthetic activity (Rusaczonek *et al*. 2015), in particular, in the protection of PA against UV radiation and other stressful factors affecting PA (Thiele *et al*. 1999, Carvalho *et al*. 2011). However, the specific mechanism is unclear.

The inactive form of PhyB ($PhyB_R$) and other types of phytochromes are synthesized in the dark. Upon absorption of red light of λ_m = 660 nm, $PhyB_R$ is transformed into the active form of $PhyB_{FR}$ (Quail 2002). Absorption of light with λ_m = 730 nm converts the $PhyB_{FR}$ form into $PhyB_R$ again. Such a reversibility, reported only for PhyB (Jiao *et al.* 2007), is in agreement with the response of the photochemical activity of PSII in WT of *A. thaliana* under irradiation with UV-A and pretreatment with RL and FRL (Table 1). The mutant *hy3* showed small protective effect of the RL pretreatment and also small reversibility of FRL which were both noticeable in WT. These findings agree with available data on the PhyB deficiency in the mutants and also support the assumption that PhyB and possibly other red/far-red reversible phytochromes are directly involved in the protection of PSII against UV-A.

As a small difference was observed between the *hy2* and the *hy3* mutants in response to UV-A, it is inferred that among all phytochromes PhyB plays the major protective role. Small RL/FRL reversibility in *hy3* (Table 1) can be explained by the presence of minor phytochromes – PhyE, PhyD, and PhyC or residual PhyB in this mutant.

We suggest that the lack of PhyB and other phytochromes shifts under oxidative stress conditions the pro-/antioxidant or ROS balance towards oxidized species in the mutants compared with the WT plants. The concept is in agreement with our data on bleaching of Chl *a* under strong UV-A (Fig. 1), in which the *hy3* and *hy2* mutants showed a faster Chl *a* bleaching compared with the WT samples. Moreover, moderate UV-A irradiation also decreased the content of photosynthetic pigments in the mutants, but not in WT. The decrease in UAPs was also stronger in the mutants compared with WT. This concept has been already discussed in our previous work (Kreslavski *et al*. 2013b), in which the overall H_2O_2 formation in the *hy2* mutant leaves was bigger than in the WT samples.

The *hy2* and *hy3* leaves were paler than the WT ones. This agrees with lower initial amount of Chls in both mutants and with decreased amount of UAPs in the mutants and can easily explain the observed higher susceptibility of both *hy2* and *hy3* mutants to strong UV-A-induced bleaching presented in Fig. 1.

There are several gene products involved in the response of higher plants to different stressful factors, among them there are chloroplastic ascorbate peroxidases and *APX1* (Koussevitzky *et al*. 2008, Maruta *et al*. 2010) as well as the chalcone synthase (*CHS*) and phenylalanine ammonia-lyase (PAL), which take part in the flavonoid biosynthesis (Weisshaar and Jenkins 1998). These genes are considered as central components of the reactive oxygen gene network of *A. thaliana* (Davletova *et al*. 2005, Asada 2006, Schmitt *et al.* 2014).

Although there was no significant enhancement of effects of RL or UV-A upon their joint action on transcription levels of genes, a role of PhyB for the regulation in the presence of both UV-radiation and RL seems to be feasible at other stages of expression of some genes. In any case, the phytochrome system generally can be involved in the protection of PSII and PA in general

Table 4. Effects of RL or RL-FRL illumination on the transcriptional activity of some genes in 26-d-old *Arabidopsis* leaves of the *hy3* mutant compared to WT and *hy2* mutant. RL – irradiation with RL for 10 min, then dark exposure for 1 h. RL-FRL – irradiation with RL for 10 min, then FRL for 10 min, after dark exposure for 1 h. Activity of WT and corresponding mutants without RL treatments are normalized to 1. Means of approx. 18 samples, $n = 6$. Symbol (-) means the absence of measurements. ^ – reliable difference between WT after RL and WT after RL-FRL, $p<0.05$. * – means the reliable increase or decrease of gene activity in samples irradiated with red light samples as compared to corresponding samples without red irradiation, $p<0.05$.

Protein	*hy3*(RL)	*hy2*(RL)	WT(RL)	WT(RL-FRL)
APX1	2.6 ± 0.3*	1 ± 0.2	3.2 ± 0.5*	1.8 ± 0.2^
sAPX	1.84 ± 0.5*	0.4 ± 0.07*	0.7 ± 0.2	0.4 ± 0.3
tAPX	1.4 ± 0.2	0.5 ± 0.1*	1.9 ± 0.25*	1.6 ± 0.4
CAB1	1.3 ± 0.3	1 ± 0.14	1.6 ± 0.3	1.55 ± 0.2
CHS	1.2 ± 0.1	0.3 ± 0.1*	1.5 ± 0.4	1.4 ± 0.2
D1	0.7 ± 0.15	1.25 ± 0.25	1.0 ± 0.2	-
PBS	0.9 ± 0.2	0.65 ± 0.2	0.95 ± 0.15	-
PhyB	1.4 ± 0.1	0.7 ± 0.2	2.0 ± 0.2*	1.9 ± 0.3
PIF3	0.85 ± 0.25	1.2 ± 0.5	1.5 ± 0.5	1.1 ± 0.3
HY5	1.2 ± 0.3	0.35 ± 0.1*	2.7 ± 0.6*	1.6 ± 0.2
HYH	0.7 ± 0.15	0.5 ± 0.2*	2.45 ± 0.35*	1.45 ± 0.06^

Table 5. Effects of UV exposure (2 h) on the transcriptional activity of some genes in 26-d-old *Arabidopsis* leaves of WT and *hy2* and *hy3* mutants. Activity of WT and corresponding mutants without UV treatments were normalized to 1. Means of approx. 18 samples, $n = 6$. *– means the reliable increase or decrease of gene activity in samples irradiated with UV light as compared to corresponding samples without UV irradiation, $p<0.05$.

Protein	*hy3*	*hy2*	WT
APX1	5.7 ± 0.6*	3.8 ± 1*	3.2 ± 0.7*
sAPX	0.5 ± 0.1*	0.8 ± 0.5	0.65 ± 0.3
tAPX	0.65 ± 0.06*	1.2 ± 0.3	0.5 ± 0.2*
CAB1	1.3 ± 0.3	0.6 ± 0.15*	0.5 ± 0.1*
CHS	2.4 ± 0.4*	1.4 ± 0.5	1.4 ± 0.4
D1	0.7 ± 0.15	1.2 ± 0.2	1.0 ± 0.2
PBS	0.5 ± 0.15*	0.34 ± 0.1*	0.6 ± 0.2
PhyB	0.6 ± 0.2	0.55 ± 0.2*	0.9 ± 0.3
PIF3	0.8 ± 0.1	0.3 ± 0.1*	0.8 ± 0.2
HY5	5.8 ± 1.1*	4.5 ± 0.9*	4.9 ± 0.7*
HYH	2.9 ± 0.7	1.9 ± 0.5	2.1 ± 0.2*

from UV-radiation by regulation of genes, such as *APX1*, *tAPX*, *CHS*, and *PPyB*.

It is therefore suggested that PhyB is the predominant RL sensor, with PhyC, PhyB, and PhyE playing a less prominent role in RL sensing. PhyA is assumed to be the most important FRL sensor (Hu *et al*. 2013). For example, Somers *et al*. (1991) have determined the levels of the mRNAs and apoproteins encoded by the *PhyA*, *PhyB*, and *PhyC* genes in *hy3* and other long hypocotyl mutants of *A. thaliana* using phytochrome type-selective monoclonal antibodies and transcript-specific probes. The authors concluded that *hy3* is severely deficient in PhyB. It was also demonstrated that *hy3* had a significant (20- to 50-fold) deficiency in PhyB and a 20- to 30-fold deficiency in the *PhyB* transcript, compared to the corresponding levels in the WT plants. However, the levels of PhyA- and PhyC-encoding mRNAs and protein levels were similar to WT.

According to our data on the response on the transcriptional activity of some genes to UV irradiation, the deficiency of both PhyB apoprotein and total phytochrome chromophore did not seem to be particularly important for the induction of the majority of the genes studied here under UV (Table 4). Both mutants showed decreased resistance of PA to UV-radiation (Table 1) and also the decreased content of pigments with small difference to each other. Therefore, the reduced content in Car and UV-absorbing pigments found in both mutants may be one of the reasons of the reduced resistance against UV-A. This agrees with our previous data: the transgenic potato plants (*Solanum tuberosum* L., lines Dara-5 and Dara-12) actively expressing the gene of the *A. thaliana* PhyB apoprotein demonstrated a higher resistance of PA to UV-B radiation compared to nontransformed plants (Kreslavski *et al*. 2015). The elevated UV-B tolerance has been suggested to be related to the increased leaf content of Car and flavonoids.

It is suggested that phytochromes affect the PA resistance not only by changing the pigment composition

but also inducing transcription activity of some antioxidant genes, such as *APX1* and *tAPX*. We suggest that along with PhyB other phytochromes also play an important role in this effect of RL, as the activity of a number of antioxidant genes is low under RL mainly in the *hy2* mutant.

Conclusion: According to our results we can summarize our main findings: (*1*) based on comparison of the responses of WT, *hy2*, and *hy3* mutants of *A. thaliana*, it was clearly confirmed that, among phytochromes, particularly PhyB contributes to a response improving the resistance against UV-A stress; (*2*) UV-A resistance in WT of *A. thaliana* can be promptly modulated by PhyB activation and/or inactivation. This finding demonstrates that rapid regulation of gene expression is involved in this process and indicates the interconnection of RL and UV-A/blue light signaling.

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