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Role of phytochromes A and B in the regulation of cell death and acclimatory responses to UV stress in Arabidopsis thaliana

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Abstract

Plants coordinate their responses to various biotic and abiotic stresses in order to optimize their developmental and acclimatory programmes. The ultimate response to an excessive amount of stress is local induction of cell death mechanisms. The death of certain cells can help to maintain tissue homeostasis and enable nutrient remobilization, thus increasing the survival chances of the whole organism in unfavourable environmental conditions. UV radiation is one of the environmental factors that negatively affects the photosynthetic process and triggers cell death. The aim of this work was to evaluate a possible role of the red/far-red light photoreceptors phytochrome A (phyA) and phytochrome B (phyB) and their interrelations during acclimatory responses to UV stress. We showed that UV-C treatment caused a disturbance in photosystem II and a deregulation of photosynthetic pigment content and antioxidant enzymes activities, followed by increased cell mortality rate in phyB and phyAB null mutants. We also propose a regulatory role of phyA and phyB in CO2 assimilation, non-photochemical quenching, reactive oxygen species accumulation and salicylic acid content. Taken together, our results suggest a novel role of phytochromes as putative regulators of cell death and acclimatory responses to UV.

Key words: Antioxidant enzymes, cell death, photosynthesis, phytochromes, reactive oxygen species, UV stress.

Introduction

In the natural environment, plant growth and development are challenged by ceaseless fluctuations of numerous factors. Plants, being exposed to diverse biotic and abiotic stresses, have developed the ability to perceive and react to both sudden and seasonal changes. One of the most unstable abiotic factors is light. In the course of evolution, plants have formed...
active mechanisms of light perception that are dependent on photoreceptors (Karpinski et al., 2003; Chen et al., 2004; Möglich et al., 2010; Chen and Chory, 2011) and photosystems (Szechynska-Hebda et al., 2010). These mechanisms allow plants to sense changes in both, the spectrum and intensity of light. Deciphering the light spectral composition enables plants to regulate the processes associated with their growth and development.

The most intensively studied plant photoreceptors are phytochromes, which are able to absorb red and far-red light. In Arabidopsis thaliana, they are represented by five isoforms: light-labile phyA and light-stable phyB–phyE (Quail, 2002). Phytochromes are synthesized in the cytoplasm as inactive precursor forms (Pr). The red light absorption triggers their conversion into active forms (Pfr) and subsequent transfer to the nucleus (Schafer and Bowler, 2002). Photoactivated phytochromes inactivate phytochrome-interacting transcription factors (PIFs), which inhibit light signalling (Khanna et al., 2004; Jeong and Choi, 2013). By direct interaction with PIFs, phytochromes induce their detachment from DNA and promote their degradation by the 26S proteasome (Park et al., 2004, 2012).

Phytochromes regulate every step of plant development and control many diverse physiological processes. Two of them, phyA and phyB, play the principal roles. They control chlorophyll synthesis and de-etiolation (McCormac and Terry, 2004, 2012). Moreover, phyA and phyB, play the principal roles. They control chlorophyll synthesis and de-etiolation (McCormac and Terry, 2004, 2012). Furthermore, it has been demonstrated that phytochromes can coordinate pathogen defence responses, participating in cross-talk between salicylic acid (SA), protein phosphatase 7 (PP7) and nucleotide-diphosphate kinase 2 (NDPK2) (Genoud et al., 2008).

Programmed cell death (PCD) is one of the most important processes regulating cell fate and thus plant growth and development. Differentiation of some tissues such as tracheary elements involves the selective elimination of viable cells. PCD is induced during various biotic and abiotic stresses (Chichkova et al., 2004; de Pinto et al., 2012). Under stress conditions, plants overproduce reactive oxygen species (ROS), which disturb cellular homeostasis and trigger cell death. On the other hand, PCD helps to maintain tissue homeostasis and enables nutrient remobilization from dying cells, thus increasing the probability of survival in adverse conditions. At the biochemical and molecular levels, plants and animals share some characteristic steps in the PCD programme, such as DNA fragmentation (Kuthanova et al., 2008), chromatin condensation (McCabe et al., 1997), and caspase-like proteolysis (Chichkova et al., 2004). However, in contrast to animals, plants possess chloroplasts in which the imbalances within photosynthetic electron transport can trigger PCD signalling pathways (Samuilov et al., 2003).

LESION SIMULATING DISEASE1 (LSD1) has been described to integrate signalling pathways in response to diverse stresses, both biotic (Rusteucri et al., 2001) and abiotic (Mateo et al., 2004; Muhlenbock et al., 2007, 2008; Wituszyńska et al., 2013b). The LSD1 mutant (lsl) is one of the best-characterized Arabidopsis mutants in the context of deregulated cell death. It demonstrates uncontrolled spread of PCD that develops under non-permissive conditions, such as infection with avirulent pathogens, a continuous photoperiod, or UV radiation (Dietrich et al., 1997; Mateo et al., 2004; Wituszyńska et al., 2015). PCD in lsl depends on ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), since null mutation in EDS1 reverts to the lsl-conditioned cell death (Rusteucri et al., 2001; Mateo et al., 2004; Muhlenbock et al., 2007, 2008; Wituszyńska et al., 2015). Moreover, the edsl mutant has been described to be less sensitive towards UV-C radiation (Wituszyńska et al., 2015).

Rapid climate changes hinder the prediction of perturbations in numerous environmental factors and their implications on ecosystems. More recent studies have focused on searching for mechanisms of UV perception in plants, since UV radiation is one of the most serious and frequent factors inducing plant PCD (Nawkar et al., 2013). UV is conventionally divided into three wavebands: UV-C (200–280 nm), UV-B (280–315 nm), and UV-A (315–400 nm). In fact, this division is somewhat arbitrary, but it is a useful contraction for considering the biological and ecological effects of different UV radiation spectra. As photoautotrophs, plants are inevitably exposed to the damaging effects of UV. It is especially harmful to photosystem II (PSII) and the CO2 assimilation process, even more than an excess of white light (Ohnishi et al., 2005). UV-C is strongly absorbed by the oxygen and ozone in the atmosphere, and in theory it should not reach the Earth’s surface. However, in the early history of the Earth, an intense solar UV-C had been a major factor limiting the evolution of early life (Paul and Gwynn-Jones, 2003; Stratmann, 2003). Moreover, UV-C-triggered changes in plant cells are comparable with those induced by UV-B radiation, which reaches Earth’s biosphere (Danon and Gallois, 1998). It has been demonstrated that mitochondria and ROS play an important role in the regulation of the plant cell death programme, induced by UV-C (Gao et al., 2008). Studies on suspension cultures of carrot cells after UV-C exposure have determined three types of cell death: necrosis, apoptotic-like PCD, and autophagy (Balestrazzi et al., 2010), suggesting that the UV-C
triggered responses are highly complex, and the interaction of different UV-induced pathways is still poorly understood.

The role of phytochromes in perceiving UV light is not well recognized, but it has been shown that phyA can mediate UV-A-dependent chloroplast gene transcription (Chun et al., 2001). Therefore, the role of phytochromes in the regulation of downstream responses to other UV spectra (UV-B and UV-C) is plausible. In this study, we formulated a hypothesis that phyA and phyB play a role in the regulation of UV-C-triggered PCD. To the best of our knowledge, this is the first study that presents such a broad characterization of phyA- and phyB-deficient mutants, focusing on photosynthetic efficiency and ROS/SA homeostasis under UV-C-induced cell death.

This study is divided into three parts. The first shows differences in photosynthesis parameters in phytochrome mutants in comparison with the wild-type plants. The second describes how phytochrome mutants react to UV-C radiation stress in terms photosynthesis and antioxidant system deregulation, and ultimately cell death. The last part shows some insight into transcriptional regulation and presents common genes involved in phyA- and phyB-mediated pathways and plant responses to UV stress.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild-type (ecotype Col-0) and three lines of phytochrome null mutants, all in the Col-0 background, were used. phyA-211, phyB-9, and double mutant phyA-211 phyB-9 previously described by Reed et al. (1993, 1994) were kindly provided by Professor E. Schäfer (Institut für Biologie II, Universität Freiburg, Germany). Additionally, lsd1 (lesion simulating disease 1) and edsl (enhanced disease susceptibility 1) mutants in the Col-0 background were used as a positive and negative control of PCD, respectively, as described previously (Wituszyńska et al. 2015). After 3 d of stratification at 4 °C, seeds were grown on Jiffy Peat Pellets (Jiffy Products International, The Netherlands) under the following conditions: 8/16 h photoperiod, 22/18 °C (day/night), relative air humidity of 70±5%, and light intensity 90±5μmol m⁻² s⁻¹. In all experiments, 3- to 4-week-old plants were used.

UV treatment

A UV-C 500 Crosslinker (Hoefer Pharmacia Biotech, USA), equipped with a Sanko Denki lamp (type G8T5, 8 W each; Japan), was used for plant UV exposure. The UV spectrum ranged from 250 to 258 nm, with the maximum at 253.7 nm. Plants were exposed to UV at a dose of 100 or 200 mJ cm⁻². Dry weight was determined for 4-week-old plants. Whole rosettes were collected, dried at 105 °C for 3 h, and each rosette was weighed.

Gas exchange and chlorophyll a fluorescence parameter measurements

Gas exchange and chlorophyll a fluorescence parameters were measured simultaneously on whole rosettes using a Portable Gas Exchange Fluorescence Systems GFS-3000 (Heinz Walz GmgH, Germany). Gas exchange parameters were measured at variable light intensity (20–1700 μmol m⁻² s⁻¹) and intercellular CO₂ concentration (ci) (60–1350 ppm) as described previously (Wituszyńska et al., 2013a). Before the first level of photosynthetically active radiation (PAR) was applied, each plant was dark adapted for 30 min. During modulated light measurements, the CO₂ concentration in the plant cuvette was maintained at 380 ppm. In modulated ci, PAR was maintained at 600 μmol m⁻² s⁻¹. Variables such as PAR, air temperature, leaf temperature, and vapour pressure deficit were recorded together with fluorescence intensity (F₀, Fₘ, Fₚ, Fₙ) and were used for calculations of gas exchange and fluorescence parameters. Following Caemmerer and Farquhar (1981), variations in gas exchange and fluorescence parameters were presented depending on PAR or ci.

For regression analysis of PAR and ci curves, the following measuring points were used: 20, 100, 300, 600, 900, 1200, and 1700 μmol m⁻² s⁻¹, and 60, 100, 165, 320, 650, and 1350 ppm, respectively. At each PAR or ci, readings of the following parameters were collected or calculated according to the manufacturer’s instructions: A, assimilation rate; ci, the intercellular molar fraction of CO₂; GH₂O, water vapour conductance; ΔΦPSII, quantum yield efficiency of photosystem II; and non-photochemical quenching (fluorescence quenched by other processes than photochemistry) measured as qN (the non-photochemical fluorescence quenching determined in relation to the maximal variable fluorescence; Schreiber et al., 1986) or NPQ (the non-photochemical fluorescence quenching determined in relation to the remaining maximal fluorescence; Bilger and Björkman, 1990). The results of assimilation rate and stomatal conductance were calculated per unit leaf area measured with a FlourCam system (PSI, Czech Republic). Statistical analysis was performed in R, version 2.12.1 using the statistics packages. To compare the regression line slopes, the procedure from Wonnacott and Wonnacott (1990) based on the use of a mute variable D was used.

Stomatal density

Imprints of the abaxial leaf side were taken using transparent glue (UHU, Germany). For each genotype, three leaves (leaves 6, 7, and 8) from three different plants were analysed. Images were taken with a confocal microscope (LSM-700; Zeiss, Germany), using the light pathway. The number of stomata mm⁻² of leaf area was calculated from three frames of each microscopic sample.

Electrolyte leakage measurement

Cell death was quantified by cellular electrolyte leakage from the whole rosettes. The assay was conducted according to Overmyer et al. (2000). Three-week-old plants were transferred to 50 ml Falcon tubes filled with 35 ml of deionized water. The conductivity of the solution (μS cm⁻¹) in relation to the rosette fresh weight (g) was determined after 1 h using a conductivity meter (InoLab Cond Level 1; WTW, Germany).

Trypan blue staining

Trypan blue staining was used to detect the level of plant cell death in situ. For each genotype, three leaves (leaves 6, 7, and 8), from three different plants were analysed. Staining was performed for non-treated and UV-treated plants, according to Idänheimo et al. (2014) with minor modifications. Freshly detached leaves were boiled with trypsin blue/lactophenol solution for 3 min and subsequently washed in 15 mM chloral hydrate solution. Samples were stored in 60% glycerol and examined by stereomicroscopy.

Hydrogen peroxide and superoxide levels determination

Hydrogen peroxide (H₂O₂) level was determined according to Velikova et al. (2000) with minor modifications. Fresh tissue (50 mg) was homogenized in TissueLyser LT (Qiagen, The Netherlands) (5 min, 50 Hz, 4°C) with 300 μl of cold 0.1% trichloroacetic acid and centrifuged at 13 000 rpm for 15 min. The supernatant was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI at a ratio of 1:1.2 (v/v/v). Absorbance was measured at 390 nm using microplate reader Multiscan-GO (Thermo Scientific, USA) and the concentration of H₂O₂ was calculated using an appropriate standard curve. Data were

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expressed as µmol of H$_2$O$_2$ per 100 mg of dry weight. The visualization of H$_2$O$_2$ in Arabidopsis leaves was performed by infiltration with 5 mM 3,3’-diaminobenzidine (DAB) as described previously (Wituszyńska et al., 2015). Nitro blue tetrazolium (NBT) staining was used to detect the production of superoxide radicals (O$_2^-$). For each genotype, three leaves (leaves 6, 7, and 8), from three different plants were analysed. Staining was performed for non-treated and UV-treated plants, according to Wohlgemuth et al. (2002) with minor modifications. Freshly detached leaves were immediately immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% NBT and 10 mM sodium azide. The leaves were vacuum infiltrated for 2 min and incubated for 2 h in the dark (without vacuum). After incubation, they were immersed in glycero/acetate:ethanol mixture (1:1.3, v/v/v) to completely eliminate chlorophyll. Superoxide production was visualized as a dark blue formazan deposit within the leaf tissue.

**Protein extraction**

Frozen leaf tissue (50–100 mg) was homogenized in TissueLyser LT (Qiagen) (5 min, 50 Hz, 4 °C) with 1 ml of extraction buffer (100 mM Tricine, 3 mM MgSO$_4$, 3 mM EGTA, 1 mM dithiothreitol, 1 M Tris/HCl, pH 7.5). Samples were incubated on ice for 15 min and centrifuged at 13 000 rpm for 20 min. The enzyme extract for the APX activity assay additionally contained 5 mM L-ascorbic acid. Protein concentration was determined using a Bradford assay kit (Thermo Scientific) with BSA as a standard.

**Enzymes activity measurements**

Spectrophotometric assay of superoxide dismutase (SOD) activity was performed according to Beauchamp and Fridovich (1971) with modifications. Enzyme assay mixture contained 0.1 M phosphate buffer (pH 7.5), 2.4 µM riboflavin, 840 µM NBT, 150 mM methionine and 12 mM Na$_2$EDTA at a ratio of 8:1:1:1:1 (v/v/v/v/v). The enzyme extract was mixed with the enzyme assay mixture in such a proportion that the inhibition of the NBT oxidation was in the range of 20–80%. Absorbance was measured at 560 nm, 15 min after sample illumination with 500 µmol m$^{-2}$s$^{-1}$ (LED Lamp SL3500-W-D; PSI) or incubation in the darkness (blank sample). Results were expressed in units (amount of enzyme that inhibited NBT photoreduction to blue formazan by 50%) (mg of protein)$^{-1}$. The activity of catalase (CAT) was measured spectrophotometrically according to Aebi (1984) with modifications. Perhydroxyl was diluted with 50 mM phosphate buffer (pH 7.0) to the absorbance of 0.5 (±0.02) at 240 nm (initial concentration of H$_2$O$_2$ corresponding to about 13 mM). The enzyme extract was mixed with 50 mM phosphate buffer in such a proportion that absorbance decreased to the range of 20–80%. CAT activity was measured as the rate of H$_2$O$_2$ decay within 2 min and calculated using the molecular extinction coefficient of H$_2$O$_2$ at 240 nm (ε=43.6 mol$^{-1}$ cm$^{-1}$). Results were expressed in µmol of H$_2$O$_2$ min$^{-1}$ (mg of protein)$^{-1}$. The activity of ascorbate peroxidase (APX) was measured spectrophotometrically according to Nakano and Asada (1981) with modifications. The enzyme extract was mixed with the assay buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 15 mM L-ascorbic acid, 40 mM H$_2$O$_2$) in such a proportion that absorbance decreased to the range of 20–80%. APX activity was measured as the rate of ascorbate decay within 2 min and calculated using the molecular extinction coefficient of ascorbate at 290 nm (ε=2.8 mmol$^{-1}$ cm$^{-1}$). Results were expressed in µmol of L-ascorbic acid min$^{-1}$ (mg of protein)$^{-1}$. Spectrophotometric measurements were performed using a UV-VIS microplate reader Multiscan-GO (Thermo Scientific).

**Salicylic acid determination**

The determination of salicylic acid (SA) was performed as described by Meeuwly and Métraux (1993). 2-Methoxybenzoic acid and 3-hydroxybenzoic acid were used as the internal standards. SA was eluted on a Luna 5 µm C18(2) 100 Å 15×4.6 mm column (Phenomenex, USA) at 30 °C for 15 min using a Shimadzu HPLC System (Shimadzu, Japan). Low-pressure gradient system was used with 20 mM phosphate buffer (pH 2.5; adjusted with 8 M HCl) and acetonitrile (75:25; v/v) at a flow rate of 1 ml min$^{-1}$. Results were expressed as µg of SA (g of dry weight)$^{-1}$.

**Chlorophyll a fluorescence measurements**

The measurement of chlorophyll a fluorescence was performed on whole Arabidopsis rosettes using the FluorCam system (PSI, Czech Republic). For both, quenching and OJIP tests, dark-adapted plants (30 min) were used to determine Fv/Fm, qN, and NPQ. However, during the chlorophyll fluorescence measurement protocol, the light-adaptation phase was also included, which enabled to determine other light-dependent parameters such as ΦPSII. Chlorophyll a fluorescence parameters have been described in details elsewhere (Baker, 2008; Wituszyńska et al., 2013a).

**Photosynthetic pigments measurements**

Frozen tissue (50–100 mg) was homogenized in the TissueLyser LT (Qiagen) (5 min, 50 Hz, 4 °C) with 1 ml of cold acetone (–20 °C). Homogenate was evaporated under N$_2$, dissolved in cold solvent A (acetonitrile:methanol:glycerol:acetic acid 90:10:1:1, v/v/v/v) and rehomogenized for 1 min. The extract was filtered (0.2 µm nylon filter; GE Healthcare, UK) and stored in the dark at 80 °C until high-performance liquid chromatography analysis (Shimadzu). Pigments were separated on a Synergy 4 µm MAX-RP 80 Å 250×4.6 mm column (Phenomenex) at 30 °C. Low-pressure gradient system was used: solvent A for 10 min to elute all xanthophylls, followed by solvent B (methanol:ethyl acetate; 68:32; v/v) for 10 min to elute the rest of pigments. The flow rate was 1 ml min$^{-1}$. Absorbance spectra were recorded at 440 nm. Pigments were identified using standards (Sigma, Hoffmann-LaRoche, Fluka). Results were expressed as peak area (mg of dry weight)$^{-1}$. The De-epoxidation state was measured as (antheraxanthin+zeaxanthin)/(violaxanthin+antheraxanthin+zeaxanthin).

**Microarray meta-analysis**

For the microarray meta-analysis, the data sets of transcripts significantly deregulated (P<0.05) in Col-phyA and Col-phyB mutants in comparison to the wild type (Col-0) were obtained from publicly available microarray results (Mazzella et al., 2005). Data were compared with the NASCARRAYS-124 microarray experiment for UV-C induced transcriptional changes (P<0.05) in Col-0 available at the NASC International Affymetrix Service. Transcriptomic data sets were functionally classified using the MapMan 3.5.0 Beta Tool (Thimm et al., 2004) in the search for various cellular pathways.

**Statistics**

If not described differently, all results were presented as means±SD of 9–12 plants per genotype from two independent experiments (n=18–24). Statistical analysis was performed in STATGRAPHICS Plus 5.1 or in R version 2.13. A Shapiro–Wilk normality test and Bartlett test of variances homogeneity were performed. For parametric tests, analysis of variance was used, while for non-parametric tests, a Kruskal–Wallis test was performed.

**Results**

**phyA and phyB are positive regulators of photosynthetic activity**

To investigate the role of phyA and phyB in the photosynthetic regulation, we used simultaneous gas exchange and
chlorophyll a fluorescence measurements for the wild-type and phytochrome mutants (phyA, phyB, and phyAB). Since phyB and phyAB Arabidopsis mutants display a constitutive shade-avoiding phenotype characterized by elongated petals (Reed et al., 1993) (Fig. 1), all the measured parameters were recalculated taking into consideration the rosette area.

Photosynthetic parameters are presented as the function of light intensity (PAR) or intercellular CO₂ mole fraction (ci) (Figs 2 and 3, Supplementary Table S2, available at JXB online). The phyA, phyB, and double phyAB mutants showed decreased CO₂ assimilation rate in comparison with Col-0 (Figs 2A and 3A). These differences were most pronounced in phyAB (Figs 2A and 3A). Stomatal water vapour conductance (GH₂O) in changing PAR and CO₂ concentration was also significantly decreased in phyB and phyAB, but not in phyA (Figs 2B and 3B). Assuming that CO₂ diffuses from air through the substomatal cavity using the same pathway in which the water vapour escapes, leaf conductance for CO₂ can be concluded from GH₂O. Due to the fact that the reduction in assimilation rates in phyA, phyB, and phyAB might be associated with lower leaf GH₂O in these mutants, we measured stomatal density in all tested genotypes (Supplementary Table S1, available at JXB online). We found significantly lower stomatal density in phyB and phyAB, which can, at least partially, explain the decreased assimilation capacity in these plants. Chlorophyll a fluorescence parameters were also changed in phytochrome mutants compared with the wild type (Figs 2C–E and 3C–E). Non-photochemical quenching parameters (NPQ and qN) that represent the excess energy dissipation by heat emission were decreased in phyA mutant in growing PAR. The same parameters measured in increasing intercellular CO₂ were affected in phyB mutants in comparison with the wild type. Significantly lower NPQ was observed in phyB. The qN parameter was decreased in phyA and phyB but increased in phyAB. Non-photochemical quenching reactions are known to diminish the efficiency of photochemistry (Graßes et al., 2002) and are major component of photoprotection (Baker, 2008). PSII operating quantum yield (ΦPSII) provides an estimate of the quantum yield efficiency of PSII in the light (Baker, 2008). In response to increasing light intensity, we did not observe any statistically important differences in ΦPSII among genotypes (Fig. 2E). On the other hand, under modulated CO₂ concentration, all phy mutants showed decreased ΦPSII (Fig. 3E).

The above data indicated that phyB and phyA plus phyB, but not phyA alone positively regulate stomata development (Supplementary Table S1) and thus affect CO₂ assimilation and stomatal conductance. Moreover, phyA and phyB influence non-photochemical reactions and PSII efficiency, but this regulation seems to be complex and condition dependent. Our results showed the genetic relationship between phyA and phyB, and their role in the mechanisms controlling photosynthesis efficiency. Since differences in photosynthetic capacity are frequently associated with altered sensitivity to environmental stresses, we tested the response of phy mutants towards oxidative stress caused by UV-C radiation.

**phyA and phyB regulates responses to UV-induced stress**

In order to study the role of phyA and phyB in the induction of cell death triggered by UV-C radiation, electrolyte leakage measurement of UV-treated versus untreated plants was performed. Two different UV doses, i.e. 100 and 200 mJ cm⁻², were used and the physiological effects of UV radiation were observed before and after UV-C stress.

Untreated control plants demonstrated no differences in ion leakage, with the exception of lsd1 mutant (Fig. 4A). lsd1 and eds1 mutants were used as a positive and negative control of UV-triggered cell death, respectively (Wituszyńska et al., 2015). UV-C radiation significantly increased the ion leakage in lsd1, phyB and phyAB (Fig. 4B and Supplementary Fig. S1, available at JXB online). The highest ion leakage for these mutants was observed 96h after the UV-C episode, in both UV-C doses. We found that phyB was most sensitive to UV-C, as it showed about 3-fold higher ion leakage 48 h after radiation with 100 mJ cm⁻² of UV-C and about 5-fold after 96h (Fig. 4B). Increasing the radiation dose to 200 mJ cm⁻² further enhanced ion leakage, but the statistically significant differences between phyB and Col-0 were observed only 96 h after UV exposure and surprisingly were less pronounced (about 1.5-fold) (Supplementary Fig. S1). These findings were confirmed by the visualization of dead cells using trypan blue staining. Our results demonstrated that, after UV treatment, most dark blue spots, which are the signs of dead cells, were present in lsd1 mutant. phyB and phyAB mutants also showed the high level of dead cells, which was consistent with the electrolyte leakage results. The lowest level of cell death

**Fig. 1.** Phenotypes of A. thaliana wild-type (Col-0), phytochrome mutants (phyA, phyB, and phyAB), lsd1, and eds1 plants in the Col-0 background. Upper panel, morphology of untreated plants; bottom panel, UV-treated plants, 96h after exposure to 100 mJ cm⁻² of UV-C.
was observed for phyA and eds1 mutants. Since the phenotypes of lsd1 and phyB are both characterized by accelerated induction of cell death, it was concluded that LSD1 and phyB can act as negative regulators of PCD. There were no differences in the ion leakage between Col-0 and phyA (Fig. 4 and Supplementary Fig. S1). Furthermore, lower level of electrolytic leakage in the double phyAB mutant, compared with phyB, may indicate the positive role of phyA in cell death regulation and an antagonistic regulatory role of phyA and phyB in this process.

In all further experiments, we used the UV dose of 100 mJ cm⁻² and measurements were performed before and 96 h after UV-C exposure. Apart from phytochrome mutants, lsd1 and eds1 were also subjected to the analysis as they demonstrate sensitivity or resistance towards UV-induced cell death, respectively (Wituszyńska et al., 2015). Qualitative and quantitative determination of foliar ROS was performed for untreated and UV-treated plants. We found that O₂⁻⁻ and H₂O₂ content differed in phy mutants even before UV-C stress (Fig. 5D, E, and Supplementary Fig. S2, available at JXB online). Non-treated phyB and phyAB exhibited 35 and 43% lower H₂O₂ content compared with control plants, respectively (Fig. 5D). This result was confirmed by DAB staining (Supplementary Fig. S2). The O₂⁻⁻ content in these mutants was also lower (Fig. 5E). In contrast, the H₂O₂ concentration in phyA was 19% higher than in the wild type (Fig. 5D), which was confirmed by DAB staining (Supplementary Fig. S2). O₂⁻⁻ in phyA demonstrated the same pattern (Fig. 5E). There was no significant difference in H₂O₂ content between lsd1 and eds1 in comparison to Col-0 before UV stress (Fig. 5D). H₂O₂ concentration 96 h after UV stress was significantly lower in all phy mutants. Compared with the wild-type plants, it was 26% decreased in phyA and 67% lower in phyB and even more in phyAB (Fig. 5D). These results were in agreement with the H₂O₂ visualization by DAB staining (Supplementary Fig. S2). 96 h after UV-C treatment, there was only little superoxide in the leaf tissue (Supplementary Fig. S3 available at JXB online), most probably because of relatively short half-life of O₂⁻⁻ (op den Camp et al., 2003) and advanced PCD expansion.
The activities of main antioxidant enzymes that keep cellular ROS homeostasis under tight control were evaluated before (0 h) and 96 h after UV-C treatment (Fig. 5A–C). We found that the deficiency of phytochromes affected the foliar levels of antioxidant enzymes both, before and after UV-C stress.

SOD activity represents the first step in the ROS scavenging pathway, detoxifying $\text{O}_2^{\cdot-}$ into $\text{H}_2\text{O}_2$ and $\text{O}_2$ (Tsang et al., 1991; Alscher et al., 2002). Before UV stress, all analysed mutants demonstrated reduced SOD activity in comparison with Col-0 (Fig. 5A). The lowest SOD activity was observed in lsd1 plants (decreased 3.8-fold). eds1 and phyAB showed comparable SOD activities, both about 50% lower than Col-0. In phyB, the enzyme activity was 32% reduced, while in phyA only a slight drop was found. Significant reduction in SOD activity measured 96 h after UV-C treatment was shown for phyB and lsd1 when compared with Col-0 (31 and 32% lower, respectively). Smaller decrease, about 16–17%, was shown in eds1 and phyAB. phyA did not demonstrate statistically significant differences (Fig. 5A).

In non-treated plants, CAT and SOD activities correlated within a particular genotype. In all mutants, CAT activity was reduced compared with Col-0 before UV-C stress (Fig. 5A). The lowest SOD activity was observed in lsd1 plants (decreased 3.8-fold). eds1 and phyAB showed comparable SOD activities, both about 50% lower than Col-0. In phyB, the enzyme activity was 32% reduced, while in phyA only a slight drop was found. Significant reduction in SOD activity measured 96 h after UV-C treatment was shown for phyB and lsd1 when compared with Col-0 (31 and 32% lower, respectively). Smaller decrease, about 16–17%, was shown in eds1 and phyAB. phyA did not demonstrate statistically significant differences (Fig. 5A).

In non-treated plants, CAT and SOD activities correlated within a particular genotype. In all mutants, CAT activity was reduced compared with Col-0 before UV-C stress (Fig. 5B). 96 h after UV-C, the highest decrease in CAT activity was observed in lsd1 and eds1, whereas the other mutants (phyA, phyB and phyAB) had 22–39% lower CAT activity (Fig. 5B). However, only in phyB mutant this difference was significant.

Similarly, the APX activity correlated with the results for CAT and SOD in non-treated plants. Before UV-C...
stress, all mutants demonstrated a decrease in APX activity compared with Col-0. However, in phyA this reduction was not significant. The highest decrease was found in lsd1 and eds1 (70–73%) (Fig. 5C). Among phytochrome mutants, the lowest APX activity in comparison with the wild type was exhibited by phyB and phyAB (around 38–44% decrease). The differences at 96 h after UV-C were less clearly pronounced. APX activity in phyA and phyAB was comparable to that in Col-0, whereas phyB, lsd1, and eds1 showed significantly lower APX activity (Fig. 5C).

The significance of interaction between SA, H$_2$O$_2$, and H$_2$O$_2$-metabolizing enzymes with oxidative damage and cell death has been presented in many studies (Mateo et al., 2006; Rivas-San Vicente and Plasencia, 2011; Petrov and Van Breusegen, 2012). Since the differences in H$_2$O$_2$ content and antioxidant enzymes activities were dependent on phytochromes, we also compared SA concentrations in mutants and wild-type plants. Untreated phyB plants had lower value of the maximum quantum efficiency of PSII ($F_{v}/F_{m}$), quantum yield of PSII ($\Phi_{PSII}$), and photochemical quenching ($q_P$) in comparison with Col-0 (Fig. 7A). phyAB mutant demonstrated increased $F_{v}/F_{m}$ and $\Phi_{PSII}$, but surprisingly its capacity for photochemical quenching was strongly decreased in comparison with the wild-type plants (Fig. 7A). phyA differed significantly from the wild type only in the higher $\Phi_{PSII}$ value. Before stress, there was no difference in the rate of excess energy dissipation through non-photochemical quenching (NPQ) between genotypes (Fig. 7A). After UV-C exposure, phyA demonstrated higher values of $F_{v}/F_{m}$, $\Phi_{PSII}$ and $q_P$ in comparison with the wild-type plants (Fig. 7A). phyB showed increased $F_{v}/F_{m}$, no alteration in $\Phi_{PSII}$, and decreased $q_P$ value. The most severe UV-triggered changes in photosynthetic parameters were demonstrated by phyAB. Double mutant showed significantly lower $F_{v}/F_{m}$, $\Phi_{PSII}$, and $q_P$ compared with Col-0 (Fig. 7A). Both phyB and phyAB had also decreased NPQ values after UV-C treatment (Fig. 7A).

**UV-C triggered changes in photosynthetic apparatus are dependent on phyA and phyB**

Imaging methods for chlorophyll a fluorescence provide a simple tool for in vivo monitoring of structural and functional state of photosynthetic apparatus, from which plant physiological status can be assumed (Gawroński et al., 2013; Wituszyńska et al., 2013a). To estimate the level of plant damage caused by UV-C radiation, we performed analyses of chlorophyll a fluorescence parameters, before and 96 h after plants’ exposure to UV-C (100 mJ cm$^{-2}$).

Untreated phyB plants had lower value of the maximum quantum efficiency of PSII ($F_{v}/F_{m}$), quantum yield of PSII ($\Phi_{PSII}$), and photochemical quenching ($q_P$) in comparison with Col-0 (Fig. 7A). phyAB mutant demonstrated increased $F_{v}/F_{m}$ and $\Phi_{PSII}$, but surprisingly its capacity for photochemical quenching was strongly decreased in comparison with the wild-type plants (Fig. 7A). phyA differed significantly from the wild type only in the higher $\Phi_{PSII}$ value. Before stress, there was no difference in the rate of excess energy dissipation through non-photochemical quenching (NPQ) between genotypes (Fig. 7A). After UV-C exposure, phyA demonstrated higher values of $F_{v}/F_{m}$, $\Phi_{PSII}$ and $q_P$ in comparison with the wild-type plants. No significant change in NPQ was observed in this mutant (Fig. 7A). UV-treated phyB showed increased $F_{v}/F_{m}$, no alteration in $\Phi_{PSII}$, and decreased $q_P$ value. The most severe UV-triggered changes in photosynthetic parameters were demonstrated by phyAB. Double mutant showed significantly lower $F_{v}/F_{m}$, $\Phi_{PSII}$, and $q_P$ compared with Col-0 (Fig. 7A). Both phyB and phyAB had also decreased NPQ values after UV-C treatment (Fig. 7A).
Phytochromes A and B regulate acclimation towards UV-C exhibited prominent but variable changes in photosynthetic parameters before and after UV-C stress. These results indicate independent and partially antagonistic involvement of phyA and phyB in photosynthetic electron transport regulation in the proximity of PSII.

A more detailed description of the role of phyA and phyB in the regulation of photosynthetic activity was performed using the OJIP test, a dark-adapted chlorophyll fluorescence technique used for plant stress measurement. All the measured parameters were presented in the radar charts as the ratios of values for individual phytochrome mutants to Col-0 (Fig. 7B). We found significant differences between genotypes in their PSII physiological states both before and after UV-C radiation.

In non-stress conditions, all of the phytochrome mutants demonstrated considerable changes in absorption flux per reaction centre (ABS/RC), trapped energy flux per reaction centre (TRo/RC), and electron transport flux per reaction centre (ETo/RC) in relation to Col-0. The ABS/RC parameter represents the ratio of the total number of photons absorbed by chlorophylls of all RCs in PSII to the number of active RCs. Before UV exposure, increased ABS/RC values were observed in all phytochrome mutants. Additionally, phyB and phyAB demonstrated higher ABS/RC ratio after UV stress, which indicated decreased number of active reaction centres in these genotypes. Increased
TRo/RC suggests that the reduction of plastoquinone QA pool was stronger in all phytochrome mutants before stress and only in phyAB after UV exposure. The ETo/RC ratio estimates the re-oxidation of reduced QA via electron transport in an active centre of PSII. Because ETo/RC is obtainable only by the active RCs, higher ratios in all phytochrome mutants before stress suggests that they had more inactive centres in comparison with the wild type. After UV-C treatment, ETo/RC for phyA, phyB, and phyAB was comparable to that of Col-0. The DIo/RC parameter characterizes the ratio of the total dissipation of untrapped excitation energy according to the number of active RCs. Strongly increased DIo/RC in phyA and phyB before UV stress and in phyB and phyAB after UV treatment indicated that the PSII RCs of these mutants were not efficient in trapping photons. Higher dissipation per active RC can also reflect worse connectivity between the heterogeneous units of PSII (Force et al., 2003). The Vj parameter, which represents the ratio of closed RCs fraction to the
proportion of the total number of RCs, was changed in phyA and phyB in non-stress conditions. Decreased $V_i$ in phyA suggested that the mutant had proportionally more open centres whereas phyB with increased $V_i$ had less, compared with the wild type. Reduction of the $Fv/Fm$ ratio indicated that the efficiency of oxygen-evolving complex (OEC) was lower in phyB before stress and in both phyB and phyAB after UV exposure in relation to the wild-type plants. Additionally, before and after UV stress, phyB and phyAB demonstrated faster accumulation of closed RCs ($M_o$) than in Col-0. All parameters expressing the quantum efficiencies, the maximum quantum yield of photochemistry (Phi_Po), the quantum yield of electron transport (Phi_Eo), and the probability that a trapped exciton enters the electron transport chain (Psi_o), were lower in phyB before UV exposure. The Phi_Po and Psi_o parameters were also significantly lower in phyB and phyAB after UV treatment. Moreover, phyA displayed higher Phi_Po and Psi_o after stress. This can suggest that the inhibition of photosynthetic electron transport observed in phyB and phyAB after UV stress may have originated from increased UV sensitivity of the light-dependent reactions (represented by Phi_Po) and reactions running behind QA (represented by Psi_o) (Fig. 7B).

Taken together, UV-C treatment resulted in a general decrease in photosynthetic efficiency, mainly in phyB and phyAB, suggesting their higher sensitivity towards UV-C stress. UV-C-triggered perturbations within the photosynthetic apparatus in these mutants might exceed their PSII repair capacity. To confirm this, further stress/recovery experiments should be performed. Nevertheless, these results point to the importance of phyB in maintaining the structural organization of PSII after stress. In contrast, the phyA mutant response to UV-C was less severe than the other genotypes. Moreover, the phyA mutation was insufficient to reverse the phyB phenotype in phyAB double mutant, suggesting a lesser role of phyA in photosynthetic apparatus functional regulation.

Significant changes in fluorescence parameters in phytochrome mutants inspired us to test the composition of their photosynthetic pigments. The average values for chlorophyll and carotenoid content, evaluated before and after UV-C stress, are presented in Table 1. Additionally, the chlorophyll $ab$ ratio was calculated (Fig. 8A). The level of chlorophyll $a$ and $b$ was not significantly changed in phyA nor in phyAB, under both control and stress conditions. In contrast, phyB demonstrated significantly lower chlorophyll content than the wild type, but this effect was observed only before UV-C treatment. These results suggest that phyB positively regulates appropriate chlorophylls concentrations in non-stress conditions. The average chlorophyll $ab$ ratio under control conditions was significantly decreased in all phytochrome mutants in comparison with Col-0. In all genotypes, UV-C radiation resulted in a considerable decrease in the chlorophyll $ab$ ratio. The highest reduction was observed in the wild-type where the chlorophyll $ab$ ratio was $~20\%$ lower than before UV stress. For phyB and phyAB, a 12.5 and 19% diminished ratio of chlorophyll $ab$ was found, respectively, whereas for phyA the ratio change was around 5%.

In control conditions, the $\beta$-carotene level was significantly lower in both phyA and phyB in comparison with Col-0 (Table 1). Interestingly, it was not statistically changed in phyAB, which suggests a complex phytochrome participation in the regulation of $\beta$-carotene content. After UV-C exposure, the $\beta$-carotene concentration did not differ among genotypes. The lutein content was not dependent on the genotype nor the stress treatment. Reduction of neoxanthin levels was observed in phyB before and after UV-C stress, and additionally in phyAB treated with UV-C.

The violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) concentrations were used to determine the de-epoxidation state (A+Z/V+A+Z) in the tested genotypes. Before UV-C exposure, the average A+Z/V+A+Z ratio was comparable to that of the wild type in phyA and phyB and was slightly lower in phyAB. Nevertheless, 96 h after UV-C exposure, phyB and phyAB showed significantly increased de-epoxidation state, while phyA significantly decreased, in comparison with Col-0 (Fig. 8B). These results indicate that phyB negatively regulates the rate of de-epoxidation, whereas phyA acts positively. Moreover, phyB seems to have more important role on A+Z/V+A+Z since the ratio in the single phyB and double phyAB mutants was similar.

**phyA and phyB commonly regulate UV-C responsive genes**

To identify genes that are commonly deregulated in phyA- and phyB-dependent signalling pathways and in the UV-C stress response, we performed a comparison of transcriptomic data available for Arabidopsis phyA and phyB with expression data for UV-treated wild-type plants. We obtained a list of 91 genes with commonly deregulated transcription levels in phytochrome mutants and UV-stressed wild-type plants (Fig. 9). A functional analysis of these genes is presented in Supplementary Table S4, available at JXB online. Within this list, we identified three genes encoding proteins

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**Table 1. Chlorophylls and carotenoids contents in untreated and UV-treated plants (96 h after 100 mJ cm$^{-2}$ UV-C dose) in wild-type plants and phytochrome mutants**

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Untreated plants</th>
<th>UV-treated plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>27.5±6.84</td>
<td>14.4±1.90***</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>9.7±2.16</td>
<td>5.4±0.68***</td>
</tr>
<tr>
<td>$\beta$-Carotene</td>
<td>7.7±1.02</td>
<td>6.2±0.60*</td>
</tr>
<tr>
<td>Lutein</td>
<td>26.1±3.59</td>
<td>24.0±7.05</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>3.4±0.31</td>
<td>3.5±0.33</td>
</tr>
</tbody>
</table>

Values are means±SD of 9–12 plants per genotype from two independent experiments ($n$=18–24) expressed as the peak area (mg of dry weight)$^{-1}$. Asterisks indicate significant differences from the wild type according to the Tukey HSD test at the level of $P<0.05$ (*), $P<0.005$ (**), or $P<0.001$ (**).
involved in photosynthetic light reactions that were highly upregulated in both phytochrome mutants and in Col-0 after UV treatment. These were two light-harvesting complex PSII proteins (LHCB4.2 and LHB1B1) and PSBO1 (PSII OEC1). The expression levels of genes responsible for hormonal signalling, especially in ABA- and auxin-dependent pathways, were also deregulated in UV-triggered plants in a phyA- and phyB-dependent manner. The ABA signalling pathway component SOT16 (sulfotransferase 16) was induced in phytochrome mutants and after UV treatment, while the negative regulator of ABA signalling AIP2 (ABI3-interacting protein 2) was suppressed in phyA and phyB but elevated in UV-treated plants (Supplementary Table S4). Moreover, two genes encoding auxin-responsive proteins - SAUR-like auxin-responsive protein and AIR9 (auxin-induced in root cultures 9) - were commonly deregulated in phy mutants and in UV-treated Col-0. We also found that an enzyme involved in vitamin B6 biosynthesis, PDX1.1 (pyridoxine biosynthesis 1.1), was overexpressed in UV-triggered plants and in both phy mutants. The expression level of VTC4 (l-galactose-1-phosphate phosphatase) involved in ascorbate biosynthesis was upregulated after UV treatment but inhibited in phyA and phyB. Furthermore, 10 transcription factors from different families were present in our list (Supplementary Table S4). Two belonging to an ethylene response factor subfamily, RAP2.1 (related to AP2 1) and RAP2.6 (related to AP2 6), were differentially regulated in phytochrome mutants and UV-triggered wild-type plants. We also found that the expression level of two genes encoding calmodulin-binding proteins - IQD14 (IQ-DOMAIN 14) and IQD30 (IQ-DOMAIN 30) - were deregulated in UV-stressed wild-type plants in a phyA- and phyB-dependent manner. These results show novel genes that may be involved in phyA- and phyB-dependent signalling pathways in response to UV. Their functional diversity casts a new light on metabolic, signalling, and molecular interactions in which these phytochromes are engaged.

Discussion

In their natural habitat, plants are constantly exposed to various stresses. The speed and accuracy of environmental signal perception as well as appropriate responses at the molecular and cellular levels underlie plant adaptation processes and enable stress tolerance. This is essential for plants’ survival, proper growth and development, and their Darwinian fitness (Külheim et al., 2002). One of the most important factor governing plant growth and development is light. The mechanisms involved in light perception and signalling are predominantly dependent on the presence of proper photoreceptors and chloroplast retrograde signalling. Among these, phytochromes receive strong attention, since they were proved to regulate different developmental plant processes. Depending on light conditions, the physiological reactions can be regulated antagonistically or synergistically by different phytochromes. phyA and phyB are examples of such a regulatory phenomenon. The rapid migration of phyA to the nucleus is promoted by very low fluences of red- and far-red light, whereas the nuclear import of phyB is activated by red light and inhibited by far-red light (Karpinski et al., 2003; Schepens et al., 2004; Franklin and Quail, 2010).
In this study, we characterized photosynthetic activity in phytochrome-deficient mutants phyA, phyB, and phyAB. The inhibition of CO2 assimilation rate and stomatal conductance in phyB and phyAB were associated with lower stomatal density (Figs 2 and 3, Supplementary Table S1). The role of phyB in CO2 uptake, transpiration rate, and stomata development has been demonstrated elsewhere (Boccalandro et al., 2009; Casson and Hetherington, 2010). Nevertheless, an additional role of phyA, in combination with phyB, as a positive regulator of gas exchange efficiency, cannot be neglected, since the decrease in CO2 assimilation, stomatal conductance, and plant growth retardation were most visible in double phyAB mutant (Figs 2 and 3, Supplementary Table S1). Moreover, the inhibition of photosynthetic activity was observed in phyB (Fig. 7). It was not related to the changes in the total absorption area of chloroplasts (Supplementary Table S1). However, it seemed to be the result of decreased levels of chlorophylls and carotenoids (Table 1). A positive influence of phyB on chlorophyll biosynthesis has recently been shown in rice (Zhao et al., 2013). Indeed, in their active forms, phyA and phyB bind to PIFs, regulating chlorophyll biosynthetic pathways (Moon et al., 2008). Here, we showed that in Arabidopsis phyB has a positive involvement not only in chlorophyll biosynthesis but also in other photosynthetic pigments. To the best of our knowledge, our study is the first report of phyB involvement in the positive regulation of carotene concentration. Lower content of photosynthetic pigments in phyB was accompanied by strong upregulation of genes encoding subunits of light-harvesting complexes: LHCb4.2 and LHCb1B (Supplementary Table S4). This may suggest the attempt of phyB plants to compensate decreased photosynthetic efficiency by overexpressing photosynthetic pigment-binding proteins. Taking together, it can be concluded that phytochromes, mainly phyB, or phyB in interaction with phyA, positively affect photosynthetic efficiency in Arabidopsis.

PCD, which is a genetically controlled cellular self-destruction mechanism in all eukaryotic organisms, can be induced either as a part of normal development or in response to various stress factors (de Pinto et al., 2012; Nawkat et al., 2013). We observed that neither phyA nor phyB was involved in cell death regulation in the absence of stress factor (Fig. 4). In contrast to the control conditions, UV-C radiation enhanced cell death in phyB and phyAB mutants compared with wild-type plants, as indicated by ion leakage measurements and staining of dead cells with trypan blue. We showed for the first time that phyB acts as a positive regulator of plant acclimation to UV stress (Fig. 4). Moreover, induced cell death in phyAB may indicate that phyA also plays some role in PCD regulation. In another study, UV-B radiation was demonstrated to improve phyB-mediated cotyledon opening during Arabidopsis de-etiolation (Boccalandro et al., 2001). These results suggest that phyB and phyA participate in signal transduction in response to UV stress.

A rapid increase in ROS production plays a key role in plant PCD induction during stress (Overmyer et al., 2003; Gill and Tutu, 2010; de Pinto et al., 2012). Isdl mutant used in our study as a control of UV-triggered PCD (Wituszyńska et al., 2015) exhibited O2˙− and H2O2 overproduction, and runaway cell death (Figs 1 and 4). Surprisingly, the content of O2˙− and H2O2 in phyB and phyAB was lower before and after UV stress in comparison with wild-type plants. A decreased content of O2˙− and H2O2 in phyB and phyAB before stress corresponded with the lower activity of SOD, CAT, and APX enzymes (Fig. 5). Thus, decreased ROS levels in phyB were not caused by higher scavenging enzymes activities. One of the reason for this phenomenon could be that the defence system in phyB is slightly activated in non-stress conditions, as seen by the higher SA levels in this mutant. After UV-C, all tested phytochrome mutants demonstrated lower H2O2, which corresponded with decreased antioxidative enzyme activities, mostly for phyB. Our results are the first indication that phyB alone and in cooperation with phyA can positively regulate ROS generation by control of the antioxidative enzymatic system. However, decreased ROS accumulation in phyB and phyAB did not restrain PCD progression in these genotypes. This fact may suggest that phyB-dependent ROS production is not a key factor involved in PCD. Therefore, ROS regulation by phytochromes is rather a part of a more subtle stress signalling during phyB protection towards UV-C stress. However, more studies need to be performed to confirm this hypothesis.

Our experiments also showed accumulation of SA in phyB before UV stress, which indicates that phyB can act as a negative regulator of SA synthesis in non-stress conditions. However, during UV stress, the SA concentration was significantly lower in all phy mutants in comparison with the wild-type plants. In this context, our results imply the existence of some unknown mechanism that leads to PCD during UV-C stress in phyA- or phyB-deficient mutants, in which H2O2/S

SA are less important than in most already known PCD-dependent pathways (de Pinto et al., 2012). Taken together, these data also demonstrate that phyB plays an important role in the regulation of UV-C-induced PCD, but independently from or with minor involvement of H2O2 and SA signalling.

UV-C radiation has been shown to negatively affect PSII efficiency (Wituszyńska et al., 2015). It has been demonstrated that photosynthetic electron transport is impaired by UV, operating within the range of 250–260 nm (for a review, see Vass et al., 2005). To analyse more precisely the role of phyA and phyB in PSII functioning after UV stress, we performed the OJIP test. Elevated flux ratios for ABS/RC, TRo/RC, ETo/RC, and D1o/RC were indicators of the plants’ higher sensitivity to drought (Wang et al., 2012), salinity (Mehta et al., 2010), cold (Liang et al., 2007), high temperature (Mathur et al., 2011), and combined salt and heat stress (Mathur et al., 2013). The OJIP results for phyB plants suggested that UV-C negatively affected the maximum quantum efficiency of PSII (ΦPi/Φo) and OEC activity (Fv/Fm) (Fig. 7B), but also the antenna structure and fate of absorbed photons. The increase in ABS/RC and D1o/RC in phyB (Fig. 7B) indicated inactivation of PSII centres after UV-C exposure. This effect was also observed in high temperature, ozone, and osmotic stress studies (Buontifetti et al., 2011; Kalaji et al., 2011; Mathur et al., 2011). All of these data suggest that the photochemical reactions were disturbed in phyB. Because of the UV-C-triggered...
damage within the photosynthetic apparatus (Wituszyńska et al., 2015), phoB was unable to use energy, harvested by light-harvesting complexes, for photochemical reactions. This could lead to photoinhibition and cell death, but was not connected to increased production of ROS compared with the wild type.

NPQ in leaves consists of three components: state transition quenching (qT), energy dependent quenching (qE), and photoinhibitory quenching (qI). qT is most important in leaves being exposed to low light levels. In non-stressed moderate to saturating light conditions, the major component of NPQ is qE, which is associated with the V+A+Z cycle. This protective mechanism is triggered by light-driven acidification of the thylakoid lumen, which induces the enzymatic conversion of xanthophyll V to and Z, and the protonation of PsbS protein in the thylakoid membranes (Müller et al., 2001). V+A+Z cycle together with another cycle, employing lutein (lutein epoxide cycle), is involved in the thermal dissipation of excitation energy in Arabidopsis (Jahns and Holzwarth, 2012). In plants exposed to severe stress restricting the consumption of reductants produced by photosynthetic electron transport, qI becomes a more significant component of NPQ (Baker, 2008). Both phoB and phoAB mutants demonstrated lower NPQ values after UV exposure. However, the de-epoxidation state in these mutants was higher after UV exposure, indicating a disturbance in photosynthetic electron transport and denoting higher susceptibility towards photoinhibition. This observation may indicate that other NPQ components such as the lutein epoxide cycle or qI were less pronounced in phoA and phoAB. Nevertheless, to confirm this presumption, further studies need to be performed.

It has been shown that chlorophylls and carotenoids associated with light-harvesting complexes can be damaged by various stress factors, resulting in reduced light-absorbing efficiency of both PSI and PSII, and hence decreased photosynthetic capacity (Vass et al., 2005; Ashraf and Harris, 2013). Carotenoids play two key roles: they serve as accessory pigments in photosystems and they protect photosynthetic apparatus against ROS, mainly singlet oxygen (Ramel et al., 2013). Lutein and β-carotene have been reported to participate in excess light energy dissipation and free radical quenching (Garcia-Plazaola et al., 2003, 2012), PSII protection against UV radiation (White and Jahnke, 2002), and PSI photoprotection under high light at chilling temperatures (Cazzaniga et al., 2012). Reduced photosynthetic efficiency and impaired PSII photochemistry, showed mainly by phoB plants, could be caused by decreased amounts of chlorophylls and carotenoids, but before UV-C treatment (Table 1). The significant reduction in photoprotective β-carotene content in phoB might also be responsible for its higher susceptibility to UV-C-induced cell death (Table 1). It has been demonstrated that severe oxidative damage in hyl1 mutant, which is defective in chlorophore biosynthesis and thus severely deficient in photosynthetic activities, was connected to a decrease in chlorophyll content and carotenoid/flavonoid metabolism and downregulation of antioxidant defences. Moreover, maximum expression of HYL1 in wild-type Arabidopsis was observed following UV-C irradiation (Xie et al., 2012). These results are consistent with our data and indicate the important role of phytochromes in UV-C stress protection and signalling. Moreover, the chlorophyll alb ratio, which is an indicator of the PSII/PSI ratio (Pfannschmidt et al., 2001), was significantly reduced in all untreated phytochrome mutants (Fig. 8A), suggesting that the number of PSII complexes in these mutants was smaller compared with the wild-type plants.

It cannot be excluded that significant developmental changes in phoB and phoAB mutants (Fig. 1) influenced their photosynthetic parameters and response to UV stress. However, it has been shown recently that pre-illumination of leaves with red light (perceived by phytochromes) has the potential to inhibit the effect of UV radiation on photosynthesis and the activity of PSII. The Arabidopsis hy2 mutant, which has reduced synthesis of phytochrome B chromophore, has been demonstrated to have decreased resistance of PSII to UV compared with the wild-type plants (Kreslavski et al., 2013). This result is consistent with our data and shows that increased resistance of photosynthetic apparatus to UV radiation involves phoB.

Expression meta-analysis revealed 91 transcripts that were commonly deregulated in phoA and phoB, and in wild-type plants after UV treatment (Fig. 9, Supplementary Table S4). High upregulation of genes encoding light-harvesting PSII complex protein and PSII OEC protein might be caused by the attempt of plants to re-synthesize compounds crucial for photosynthetic apparatus, compensating effects of photosynthetic pigments loss after UV treatment. These phoA- and phoB-dependently expressed proteins can be considered as the most UV-susceptible photosynthetic apparatus components. The overexpression of gene encoding PDX1.1 (pyridoxine biosynthesis 1.1), the enzyme involved in vitamin B6 biosynthesis, was common for UV-triggered plants and both pho mutants. Recent studies have demonstrated that vitamin B6 in Arabidopsis participates in the resistance to abiotic stresses (Havaux et al., 2009; Vanderschuren et al., 2013). The lower expression level of gene encoding ascorbate biosynthesis enzyme VTC4 is consistent with decreased APX activity, especially in phoB. We also found two transcription factors belonging to the ethylene response factor subfamily, RAP2.1 and RAP2.6, that were differently regulated in phytochrome mutants and UV-triggered wild-type plants. Both of these have been shown to play an important role in defence responses to various abiotic stresses (Dong and Liu, 2010; Zhu et al., 2010). Two members of the IQD family of proteins that bind calmodulin were downregulated in phoB mutants and in the UV-treated wild type. This suggests that some involvement of calcium signalling is common for phytochrome- and UV-dependent signalling. Furthermore, we identified other genes whose products are involved in the phyA- and phoB-regulated pathways during the response to UV radiation such as: protein kinases (MPK2), proteins involved in ABA, auxin, and calcium signalling, and transcription factors from the MYB, zinc finger, and bZIP families.

Conclusions

The results presented in this work indicate an important role of phytochromes in the control of light-mediated photosynthetic activity and UV-C-induced PCD. phoB and phoA seem
to be complex regulators of photosynthesis, starting from the gene expression level - by adjusting PSII photochemistry and ROS/SA signalling level - by to photosynthesis efficiency determined at the whole-plant level. Our results shows that phyB is a positive regulator of photochemical reactions in PSII and photosynthetic acclimatory responses to UV-C-induced stress. However, an auxiliary/complementary effect of phyA as the interacting partner of phyB is also evident. Because UV has been shown to induce damage of PSII and entire chloroplasts similar to that observed by exposure to excess light (Ohnishi et al., 2005), we suggest that the processes extinguishing excessive energy and preventing subsequent PCD in response to impaired photosynthetic electron transport depend on phyB and phyA.

**Supplementary data**

Supplementary data are available at JXB online.

**Supplementary Fig. S1.** Cell death, expressed as the ion leakage, determined for UV-treated plants with dose 200 mJ cm\(^{-2}\).

**Supplementary Fig. S2.** Hydrogen peroxide concentration visualized for untreated and UV-treated plants, 96h after UV exposure (100 mJ cm\(^{-2}\)) using DAB staining.

**Supplementary Fig. S3.** Superoxide visualized in UV-treated plants, 96h after UV exposure (100 mJ cm\(^{-2}\)) using NBT staining.

**Supplementary Table S1.** Dry weight, stomatal density, and chlorophyll autofluorescence of wild-type and phytochrome mutants.

**Supplementary Table S2.** Statistical analysis of gas exchange and chlorophyll \(a\) fluorescence parameters in response to different light intensities or different CO\(_2\) concentrations.

**Supplementary Table S3.** Statistical analysis of chlorophyll fluorescence parameters measured via the OJIP test.

**Supplementary Table S4.** Expression characteristics of genes commonly regulated in phyA and phyB mutants, and in wild-type plants after UV exposure.

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