Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 66, No 4, 2013

BIOLOGIE

Biophysique

EFFECTS OF 24-EPIBRASSINOLIDE PRE-TREATMENT ON UV-B-INDUCED CHANGES IN THE PIGMENT CONTENT OF PEA LEAVES

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(*Submitted by Academician K. Koumanov on December 15, 2012*)

Abstract

In the present work, the effects of 24-epibrassinolide (EBR) on the UV-B-induced changes in the pigment content of pea leaves were studied. Control (non-EBR-treated) and EBR-treated plants were irradiated with UV-B for 3 h and pigment analysis was performed after 24 and 48 h. The results show that EBR spraying of plants 48 h prior to UV-B exposure alleviates its detrimental effect on chlorophyll *a* and *b* (Chl *a* and Chl *b*) content in comparison with control pea leaves. An increase in carotenoids (Car) and UV-B absorbing compounds was also observed at low dose of UV-B radiation. For the first time, it is shown that UV-B damage effect on control leaves is accompanied by a significant (more than 50%) increase in their pheophytin *a* (Pheo *a*) content 48 h after the UV-B exposure and that the EBR pre-treatment prevents the increase of Pheo *a* content in UV-B irradiated leaves. In addition, it is demonstrated that EBR application modifies UV-B-induced alterations of energy distribution between the main pigment-protein complexes in pea thylakoid membranes.

Key words: brassinosteroids, UV-B radiation, photosynthetic pigments, pheophytin *a*, UV-B absorbing compounds, 77 K fluorescence

Introduction. Brassinosteroids are steroidal plant hormones involved in a wide range of developmental processes as well as in plant responses to environmental stresses via activation of different protective mechanisms (for review see [1]). To date the mechanisms by which these compounds are involved in plant stress responses are not clear. It has been shown that the EBR application exerts a direct role in the regulation of plant photosynthesis [2]. Several studies

have also reported the role of EBR in the protection of photosynthetic apparatus against environmental stresses by enhancing plant tolerance to abiotic stresses [1, 3–5]. Brassinosteroid-enhanced stress tolerance was proposed to relate to increased activity of the reactive oxygen species scavenging system – antioxidant enzymes (via accumulation of abscisic acid) and with increased content of ascorbic acid and carotenoids [1, 3, 5]. However, the participation of EBR in the protection against UV-B stress is far from completely understood. In particular, the influence of brassinosteroids on the UV-B irradiation-dependent gene expression in *Arabidopsis* mutants, defective in the biosynthesis pathway of EBR, has been investigated and it has been assumed that involvement of EBR in UV-induced gene expression occurs at low doses of radiation [6].

It is well documented that UV-B radiation exhibits harmful effects on whole plants and plant cells [7]. UV-B radiation has multiple targets as exposure to enhanced levels of UV-B results in DNA damage, impaired photosynthesis, destruction of proteins, lipids, quinones and pigments as well as diversion of metabolic energy towards defence mechanisms. Importantly, responses to UV-B are mediated by photomorphogenic signalling, which stimulates the expression of genes involved in UV-protection and hence promotes plant survival under UV-B radiation. These defence mechanisms include production of UV-B-absorbing compounds, DNA repair and increased antioxidant activity (see [7]). The content of chlorophylls, carotenoids and UV-B absorbing compounds is frequently used as indicator of plant sensitivity to UV-B radiation ([8] and refs therein). It has also been demonstrated that the UV-induced effects on photosynthesis and pigment content depend on the radiation doses and duration of treatment [8, 9].

The objective of this report was to evaluate the effect of exogenously applied EBR against UV-B-induced alterations in the pigment composition and energy interaction between both photosystems.

Material and methods. ***Plant growth and treatment.*** Pea plants (*Pisum sativum* L. cv. RAN1) were grown as water culture at controlled conditions under light intensity 100 µmol photons m^{-2} s^{-1} with 10 h light/14 h dark photoperiod at room temperature. Pea plants (12-days old) were sprayed with 0.1 mg L^{-1} 24-epibrassinolide (EBR, Sigma–Aldrich) or distilled water as described previously [3]. 48 h after EBR application, the plants were subjected for 3 h to UV irradiation at room temperature and then transferred back to control light/dark conditions and kept for 24 and 48 h. As a source of UV-B irradiation, fluorescent tubes with emission at 312 (±25 nm) (TL 20W/12 R, Philips, Hamburg, Germany) were used. The biological effectiveness of UV-B radiation was 14.4 kJ m^{-2} d^{-1} (aprox. 0.3 W m^{-2}, i.e. low dose).

Pigment analysis. Photosynthetic pigments were extracted from pea leaves (50 mg) with 80% acetone, clarified by centrifugation and the absorption spectrum of supernatant was immediately recorded with spectrophotometer SPECORD 210 PLUS, Edition2010 (Analytik–Jena AG, Germany). Chl *a* and *b* and Cars

contents were determined according to LICHTENTHALER [10]. The Pheo *a* content was determined using the equations proposed in [11] for pigment quantification in photosystem II (PSII) reaction centre preparations and corrected according to HPLC-established Chl *a*/ Pheo *a* molar ratio for leaf-pigment extracts from various species [12]. This correction was applied besause we established that for leaf extracts this method [11] gives values for Chl *a* coinciding with the values determined by the method of Lichthentaler [10], but the Pheo *a* content was overestimated. Anthocyanins and UV-B absorbing compounds were quantified according to [13] with some modifications. Pea leaves (50 mg) were homogenized in 6 mL of medium containing methanol:HCl:H2O (79:1:20), centrifuged, and anthocyanin levels were estimated from the methanolic extracts as A_{535}–$0.24A_{653}$. Concentrations of "total" UV-B absorbing compounds were estimated from 50-fold dilutions of the methanolic extract as A_{300}.

Low temperature fluorescence measurements. Fluorescence emission spectra at low temperature (77 K) of isolated thylakoid membranes from pea leaves were recorded as in [14]. Fluorescence was excited at 436 nm and registered in the region 660–780 nm with slit widths of 4 nm. Thylakoid membranes were isolated from control and treated leaves 24 h after UV-B irradiation as described previously [15] and resuspended in medium: 40 mM HEPES (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose to a concentration of 15 µg Chl mL^{-1}.

Results and discussion. In this study, pea plants were sprayed with 0.1 mg L^{-1} EBR 48 h before starting of UV-B treatment since it was previously shown that EBR has greatest effect under these conditions [2, 3]. The effects of EBR on the plant tolerance to UV-B exposure were evaluated by changes in the content of the main photosynthetic pigments (Chl *a*, *b*, total Car) and in the content of anthocyanins and UV-B absorbing compounds, measured 24 and 48 h after irradiation. The contents of Chl *a*, *b* and Car, expressed per gram fresh weight, are included in Table 1. Data presented show that the contents of these pigments are affected by low dose of UV-B treatment. It can be seen that the amount of Chl *a* and Chl *b* is slightly decreased in control samples. Chl *b* is more affected by UV-B radiation than Chl *a*, which results in an increase of the ratio Chl *a*/*b*. These observations are in agreement with previously reported data about the effect of UV-B on pigments [7, 9], indicating a preferential destruction of Chl *b* biosynthesis and/or degradation of precursors upon UV-B exposure. Comparison between the control and EBR-treated plants, subjected to UV-B irradiation, indicates that exogenous application of EBR has a protective effect against UV-B-induced pigment destruction, Chl *b* being more protected by EBR than Chl *a*, thus leading to a decrease of Chl *a*/*b* ratio (Table 1). Results indicate that EBR-treatment protects the content of Chl *a* by 12% and that of Chl *b* by 26%, measured 48 h after exposure to UV-B radiation. The Car content slightly increases 48 h after UV-B treatment of control plants. EBR treatment itself results in an increase (by 11%) of carotenoid content in leaves at non-stress

Table 1

The effect of pre-treatment with 0.1 mg.L^{-1}EBR on the pigment content of pea leaves before and after UV-B irradiation for 3 h. The pigment analysis was performed 24 and 48 h after UV-B exposure. Pigment contents are expressed as µg per grams fresh weight (FW) calculated according to Lichtenthaler [10]. Values are mean (± SE) from 2 to 3 independent experiments

	Pigments (µg g^{-1} FW)				
	Chl *a*	Chl *b*	Chl *a*/*b*	Car	Pheo *a**
no UV-B					
Control	1948 ± 25	676 ± 31	2.88	538 ± 39	15.8 ± 2.8
EBR-treated	2092 ± 63	660 ± 22	3.17	597 ± 37	16.2 ± 2.9
% of Control	107	98	110	111	102
24 h after UV-B					
Control	1849 ± 55	594 ± 47	3.11	581 ± 27	12.1 ± 2.2
EBR-treated	2020 ± 32	667 ± 20	3.03	686 ± 45	11.5 ± 2.1
% of Control	109	112	97	118	95
48 h after UV-B					
Control	1795 ± 98	564 ± 40	3.18	592 ± 50	26.2 ± 4.7
EBR-treated	2007 ± 83	714 ± 31	2.81	707 ± 61	14.3 ± 2.6
% of Control	112	126	88	119	54

* Pheophytin *a* content was determined as described in Materials and methods section.

conditions. After UV-B irradiation, Car content increases more strongly in EBR pre-treated leaves in comparison with non-treated ones (Table 1), which indicates the involvement of EBR in protection of leaf pigments from destruction as well.

The long-known visual leaf symptom of UV-B damage for pea leaves (bronzing/browning) (e.g., [16]) was clearly visible as progressive browning of the control leaves, but not of EBR-treated ones upon increasing the days after UV-B exposure (images not shown). In order to test whether a destruction of the main photosynthetic pigments accompanied the browning of non-EBR treated leaves, normalized absorption spectra of the leaf pigment extracts were compared (Fig. 1). It is clearly seen that the spectrum of the extract from control leaves 48 h after UV-B exposure shows most significant difference in the shape. This spectrum exhibits enhanced absorption at ∼ 415 nm and 537 nm. Since these spectral features are characteristic for Pheo *a* (e.g., [11]), the results indicate a significant enhancement of Pheo *a* content in this leaf extract (Fig. 1 and Table 1). It is worth noting that Pheo *a* content in control and EBR-treated leaves without UV-B irradiation is almost the same, while 48 h after UV-B irradiation accumulation of Pheo *a* in the control leaves is markedly increased. In EBR-sprayed leaves, the Pheo *a* content was not influenced by the UV-B radiation even 48 h after treatment (Table 1). These results are the first evidence for the ability of EBR to preserve pea leaves from an increase of the Pheo *a* content after a low dose of

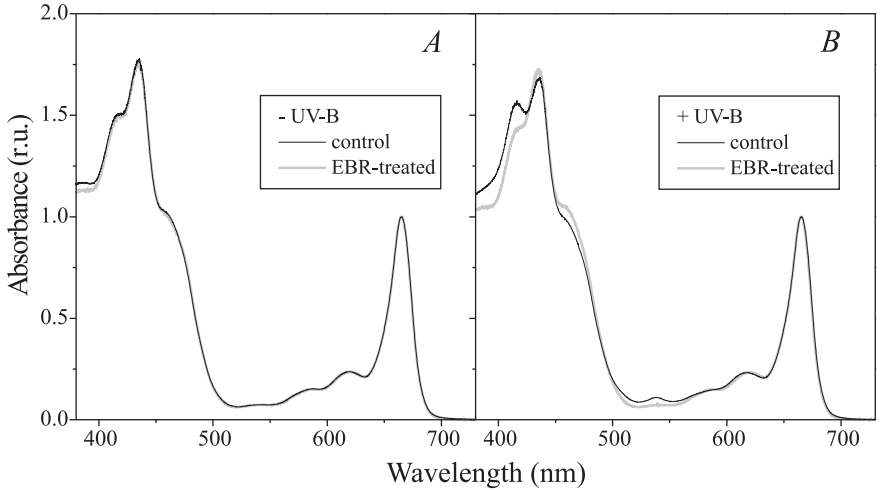

Fig. 1. Absorption spectra of 80% acetone pigment extracts from non-irradiated (A) and 48 h after UV-B irradiation for 3 h (B) control (thin black line) and EBR-treated (thick grey line) pea leaves. The spectra are normalized to the maximum in the red region

UV-B radiation. Since Pheo a is very easily formed from Chl a in an acidic environment, we can speculate that under our experimental conditions a certain local acidification takes place, which promotes formation of Pheo a in UV-B irradiated control leaves and that the treatment with EBR prevents this process. On the other hand, the observed browning of UV-B-treated leaves could be due to the formation of oxidized polyphenol products [17]. Recently, EBR ability to reduce the polyphenol oxidase activity under stress has been reported [18]. In the same line of reasoning, we can suppose that EBR prevents the UV-B-induced browning of leaves.

The content of UV-B protective compounds after UV-B irradiation was also determined. In Figure 2A data about UV-B absorbing compounds are presented measured 24 and 48 h after irradiation. As it can be expected, as a response to UV-B irradiation, an increase of UV-B absorbing compounds is observed, more pronounced in non-EBR treated plants. These compounds could be involved in protective mechanisms such as UV-screening or could serve as stress markers giving information about the extent of UV-B damage. In EBR pre-treated leaves, the level of UV-B absorbing pigments is lower in comparison with non-EBR-treated plants (Fig. 2A) which might indicate an increased UV-B tolerance after exogenous application of EBR. In Figure 2B the UV-B-induced changes of anthocyanin levels in pea leaves are presented, measured 24 and 48 h after irradiation. It is seen that pre-treatment of pea plants with EBR does not influence the anthocyanin amount in non-irradiated leaves. UV-B irradiation results in an increase of anthocyanin level, more pronounced after 24 h. Control and EBR-

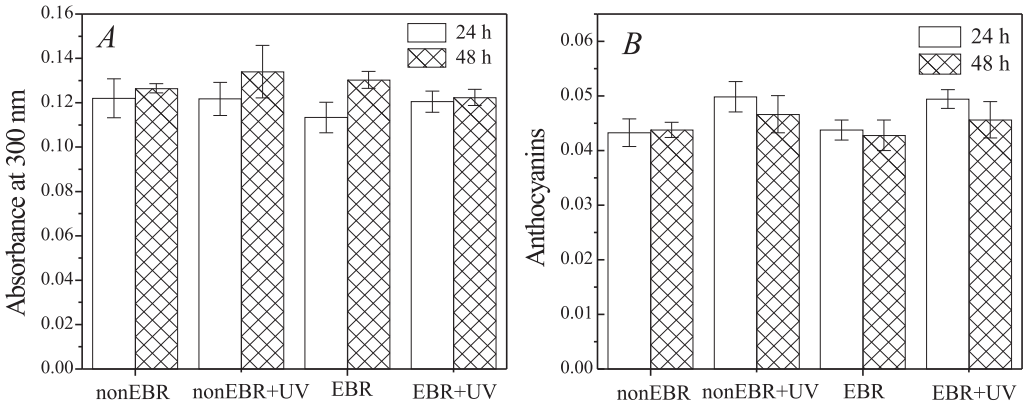

Fig. 2. Effect of UV-B irradiation for 3 h at room temperature of pea plants – control and treated with 0.1 mg L^{-1} EBR on: (*A*) UV-B absorbing compounds and (*B*) anthocyanins. UV-B absorbing compounds and anthocyanins were measured 24 h (blank columns) and 48 h (patterned columns) after UV-B irradiation

treated plants show almost the same increase of anthocyanins as a result of low dose of UV-B irradiation.

It is well documented that UV-B radiation has adverse effect not only on pigment-protein complexes of the photosynthetic apparatus, but also on the thylakoid membrane organization (see [7]). In order to verify the effect of EBR on the UV-B-induced structural alterations and/or reorganizations of the pigment-protein complexes in thylakoid membranes, we investigated changes in the 77 K fluorescence emission ratios (F685/F695 and F735/F685) measured in isolated thylakoid membranes from control and leaves treated with EBR. The ratio F685/F695 is informative about the energy interaction in the complex of PSII and its proximal antenna, while F735/F685 characterises the energy delivery to PSI (see [14]). The values for F685/F695 ratio of all samples did not show any alterations (Table 2) indicating that the energy distribution in the PSII-antenna complexes was not influenced by treatment with EBR and UV-B radiation. The observed slight increase of the F735/F685 ratio after UV-B radiation, with about 5% for thylakoids isolated from leaves non-treated with EBR and with around 11% for those treated with EBR (Table 2), is due to more energy delivered to PSI, i.e. to unstacking of the thylakoids [14, 15]. Data show that EBR pre-treatment leads to stronger UV-B-induced increase of the F735/F685 ratio under our experimental conditions. This could be interpreted as an EBR-induced defence mechanism against UV-B damage of the photosynthetic apparatus, since PSII centres in grana membranes are more UV sensitive than those in stroma lamellae [19]. Recently, it has been reported that EBR treatment induces accumulation of abscisic acid (ABA) as a plant adaptive response to high and low temperature stress (see [5]). On the other hand, Maslenkova et al. [20] have reported that high level of ABA

Table 2

Fluorescence ratios, F685/F695 and F735/F685, calculated from 77 K fluorescence emission spectra of thylakoid membranes isolated from control (non-EBR-treated) and pea plants treated with EBR, non-irradiated and irradiated with UV-B for 3 h at room temperature. Ratios were calculated after subtraction of baseline. Values are mean (± SE) from 4 independent experiments

	F685/F695	F735/F685
no UV-B		
Control	1.30 ± 0.03	1.28 ± 0.07
EBR-treated	1. ± 0.02	1.31 ± 0.08
after UV-B		
Control	1.27 ± 0.03	1.34 ± 0.08
EBR-treated	1.28 ± 0.05	1.42 ± 0.13

induces structural reorganizations of thylakoid membranes and raises the amount of stromal thylakoids. It can be speculated that the observed unstacking (i.e. increased F735/F685 ratio, Table 2) after UV-B irradiation of EBR pre-treated plants is probably due to the accumulation of ABA.

Conclusions. Data presented show that pre-treatment of pea plants with 0.1 mg L^{-1}EBR modifies the response of plants to low dose of UV-B irradiation. Exogenous application of EBR by spraying of plants for 48 h before UV-B exposure protects photosynthetic pigments against UV-B-induced destruction, Chl *b* being protected to a higher extent than Chl *a*. It is shown for the first time that UV-B irradiation results in an increase of Pheo *a* content and that pre-treatment with EBR abolishes this increase. In addition, the UV-B-induced increase of total flavonoids is reduced in EBR-treated plants. Concomitantly, a redistribution of excitation energy in favour of PSI, better expressed in EBR-treated plants, is observed but almost no changes in energy interactions in PSII-antenna complexes are detected. In summary, the results of the present study demonstrate that EBR-induced tolerance to UV-B irradiation most probably involves structural changes in thylakoid membranes and enhanced biosynthetic pathway of carotenoids, which protect the chlorophylls from photodestruction.

Acknowledgements. This work was supported by the Bulgarian Academy of Sciences. The authors are thankful to Dr S. Krumova for kindly providing EBR.

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