PD-131625

Multiple role of peroxidase enzymes in plant acclimation to UV

Closing report

The main aim of project PD-131625 was to investigate the complex role of class III phenolic peroxidases (POD) in leaves acclimated to ultraviolet radiation (UV, 280-400 nm). Although access to the university facilities was strongly limited for a specific period due to the SARS-Cov-2 pandemic, the project fulfilled its goals. That limitation slightly affected the original schedule of the research plan: instead of growth chamber experiments, a series of outdoor studies have been performed in order to characterize the regulating effects of ultraviolet radiation.

RESULTS

1. Functional heterogeneity of plant phenolic peroxidases

Our previous study emphasized the selectivity of plant phenolic peroxidases (PODs) to different artificial substrates. Moreover, these substrates showed different sensitivity for UV-B triggered responses in tobacco leaves (Rácz et al. 2018, J. Plant Physiol. 221, 101-106.). Based on these preliminary results, we tested the role of natural POD substrate leaf phenolics occurring in tobacco leaves. Four-week old wild type tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants were exposed to 6.9 kJ m^{-2} d^{-1} (biologically effective dose) supplemental UV-B radiation or either treated by exogenous hydrogen peroxide (H_2O_2) in growth chambers. According to the chlorophyll fluorescence analyses, neither of the treatments ended in stress for the plants, therefore, acclimative responses could be reported. Besides the investigation of phenolic substrate specificity of PODs, further H_2O_2-related responses have been also analyzed. Although both UV-B or H_2O_2 treatments resulted in elevated H_2O_2 concentration in tobacco leaves, the pattern of the H_2O_2-related responses was different. While ascorbate peroxidase (APX) or catalase (CAT) activities were increased rather by UV treatment, non-enzymatic H_2O_2 scavengers were accumulated in H_2O_2-treated leaves. The main groups of UV-responsive leaf phenolics have been identified by thin layer chromatography and have been tested as H_2O_2 antioxidants and POD substrates. These compounds contribute to H_2O_2 balancing in a different manner: quercetin glycosides are more effective H_2O_2 scavengers, chlorogenic acid rather acts as a substrate for phenolic peroxidases. In this study, we demonstrated that leaf H_2O_2 enhancer UV-B probably affects specific POD responses, and highlighted the existence of unique, UV-specific acclimatory pathways.

The work was completed prior to the COVID-19 outburst and published in 2020 in Scientific Reports, an open access D1 journal.

2. Contribution of specific UV wavelengths to plant development under natural conditions

Due to the unforeseen lockdown at the University of Pécs and the limited access to the laboratory instruments, a part of the project experiments was carried out under natural light conditions at our open field experimental site instead of in growth chambers. Depending on the ambient weather conditions, tobacco (*Nicotiana tabacum*) (spring-summer) or thale cress (*Arabidopsis thaliana*) (autumn) model plants were used for characterizing leaf photosynthetic and antioxidant responses under natural UV exposure. During late-spring and summer periods, wild-type tobacco (Wisconsin 38, W38) plants were grown under a series of specific glass UV filters. Since the filters were transparent to different wavelengths of ultraviolet radiation, the plants were exposed to different doses of natural UV besides photosynthetically active radiation (PAR). Plant growing outdoors was cumbersome at some point due to the unexpected biotic (snails, aphids) or abiotic (wind or heavy rain) challenges.

The acquisition of glass filters used for the project work was covered by another NKFIH grant. Prior to the availability of Schott glass filters, a preliminary study on tobacco plants was carried out using three kinds of plastic filters: i) removing the whole ultraviolet spectrum, ii) blocking the UV-B part of sunlight, iii) transmittant to the photosynthetically active radiation (PAR) and UV, too.

Tobacco's morphological responses, pigment content, and CO_2-fixing capacity have been recorded *in situ*, then PSII efficiency was characterized by chlorophyll fluorescence parameters. Despite the presence of UV-B radiation resulted in reduced plant size and leaf area, the biomass of the leaves seemed independent of light conditions, most likely due to the leaf thickening effect of UV. Elevated photosynthetic parameters like PSII quantum yields (Fv/Fm, Y(II)), stomatal conductance (gsw), or CO_2 uptake rate (A) suggested that the leaves developed under PAR + UV-A conditions were able to operate photosynthesis in a more efficient way. Pigment biosynthesis was also affected by UV: under UV-B excluding conditions, the synthetic pathway has completed and thus resulted in elevated anthocyanin concentrations, while the presence of UV-B radiation caused flavonoid accumulation, which indicates the partial retention of biosynthesis at a mid-stage. Phenolic peroxidase activity followed the accumulation pattern of its potential substrates, so the POD activation went in parallel with the increasing UV doses.

In order to a more complex analysis of natural ultraviolet spectra to plant developmental processes, seven specific glass UV filters were installed at our outdoor experimental site. The filters are transparent above the following wavelengths: 280, 295, 305, 316, 320, 324, and 395 nm. Wild-type tobacco plants were developed under these filters in a summer period (n=5).

Besides the unaffected maximal PSII efficiency (F_v/F_m), net photosynthetic rates, stomatal conductance, and light-adapted quantum yields were lower in leaves developed under filters transmittant to high energy UV-B photons (below 305 nm). That highlights the

limitations of F_v/F_m as a generally accepted stress parameter and suggests a deeper analysis of photosynthetic responses to a complex interpretation of acclimative responses.

In addition to the affected photosynthetic responses, we detected decreased leaf size and increased flavonoid content in direct proportion to the UV exposure. The HPLC profiling showed that the main phenolic components of Wisconsin 38 tobacco leaves are the following: chlorogenic acid, quercetin rutinoside, cryptochlorogenic acid, neochlorogenic acid, kaempferol rutinoside, *p*-coumaric acid, and quercetin glucuronide in decreasing order. Among these, chlorogenic acid, quercetin rutinoside, kaempferol rutinoside, and *p*-coumaric acid are the most responsive to increasing UV exposure. On contrary, neochlorogenic acid was in higher amounts in leaves developed under the lack of UV-B. As we reported in Rácz et al. (2020), chlorogenic acid is preferably used as a substrate for POD enzymes instead of for direct ROS scavenging, while quercetin rutinoside is a more effective antioxidant. The antioxidant pattern (SOD, POD anti-$^{\bullet}$OH) of W38 leaves suggested elevated cellular hydrogen peroxide concentrations among a UV dose gradient. We hypothesize that the elevated H_2O_2 concentration emphasizes its role as signalling molecule under natural UV conditions, i.e. in stomatal closure.

2.1 The contribution of the UV-B photoreceptor to plant acclimative responses under natural conditions

Due to the limited dimensions of filters (i.e. 40 x 40 cm for glass filters), developmental responses of wild-type thale cress (*Arabidopsis thaliana* L. cv. Wassilewskija) plants were also studied under the same conditions, in higher replications, accompanied by a mutant genotype lacking its UV-B photoreceptor (*uvr8-7*). By using a UVR8 mutant, we were able to study the UV-B photoreceptor dependency of specific responses at the same time.

Similar to tobacco studies, UV-A radiation triggered more efficient photosynthesis in Arabidopsis as well, indicated by the elevated electron transport rates (ETR) or light-acclimated PSII quantum yields (Y(II)). ETR rates were lower in the mutant plants were developed either under a filter transparent to full sunlight or under UV-excluding conditions in comparison to the related WTs. The former difference may originate from the altered energy dissipating pathways found in *uvr8-7* plants developed under UV-B transmitting filters. Less effective ETR capacities available in *uvr8-7* mutants in response to UV-B radiation led to more effective non-photochemical quenching (NPQ). Whilst the ETR or the NPQ values were affected by the UVR8 mutation or light condition, the PSII trapping efficiency of leaves was independent of either genotype or treatment, and thus suggests a sufficient energy transfer from LHCII to PSII reaction centres in mutant plants, too. Therefore the detected differences in NPQ values probably came from a different xanthophyll cycle available in *uvr8-7* under UV-B exposure. In order to find it out, HPLC analysis was performed from freeze-dried leaves in collaboration with Prof. Marcel Jansen and his group at University College Cork, Rep. of Ireland.

HPLC results showed a significant decrease in tocopherol or VAZ content (violaxanthin, antheraxanthin, zeaxanthin) in Wassilewskija plants under UV transmitting filters. In addition, the presence of UV-B radiation caused a ca. 20% drop in carotenoid concentration in both WTs and mutants. Xanthophyll content was also affected similarly in *uvr8-7* plants, but the total amount of VAZ in these plants was 20-25% higher independently of growth conditions.

Via a non-invasive method, epidermal flavonoid and anthocyanin indexes were also recorded *in vivo*. Flavonoid accumulation in Ws leaves increased by the elevating UV ($p < 0.001$), while in *uvr8-7* plants increasing UV doses affected flavonoid concentrations opposite ($p < 0.001$), and caused lessened flavonoid content in leaves exposed to the full solar spectrum (Fig, 1).

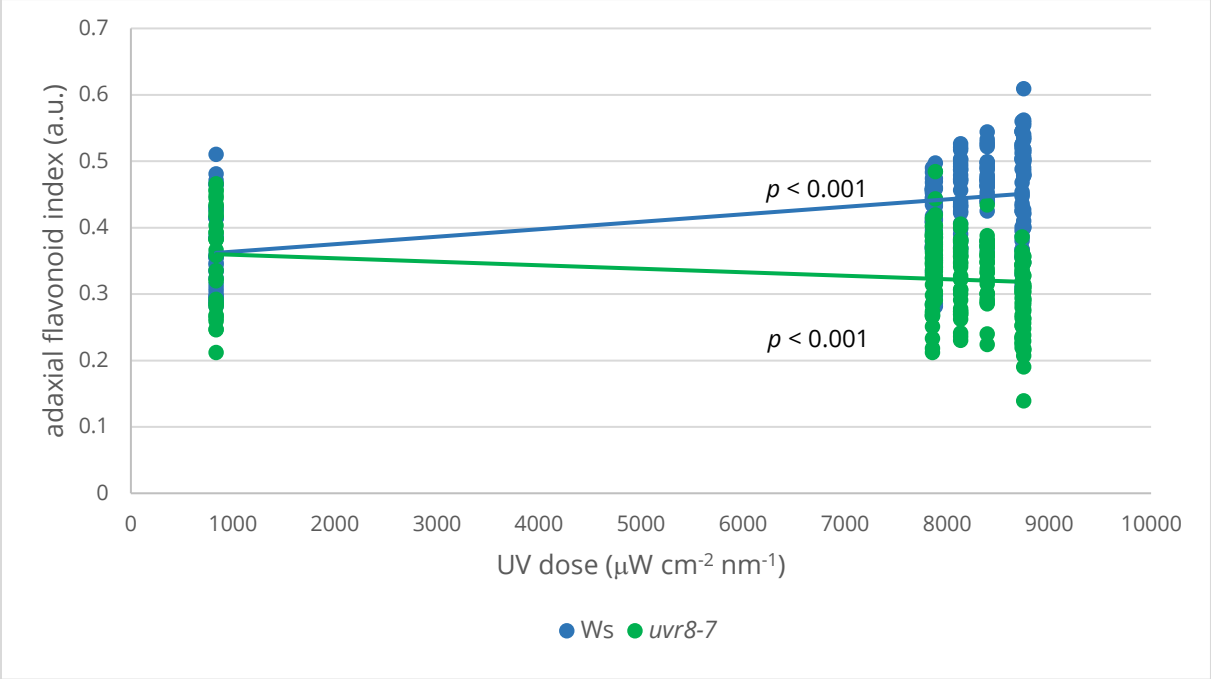

Figure 1. The adaxial flavonoid index of two *Arabidopsis thaliana* genotypes: Wassilewskija wild-type (Ws) and *uvr8-7*. Dots represent leaf flavonoid values measured under different UV environments (blue, Ws; green, *uvr8-7*) and straight lines show results of linear fits for data points (n=32). The *p* values characterize the statistical significance of linear UV dose dependence.

The presence of shorter UV-A wavelengths (below 324 nm) significantly increased the adaxial anthocyanin indexes in both genotypes, and no further elevation was recorded under UV-B transparent filters, so this result suggests that anthocyanin biosynthesis is independent of UV-B radiation and UVR8 photoreceptor, too (Fig. 2).

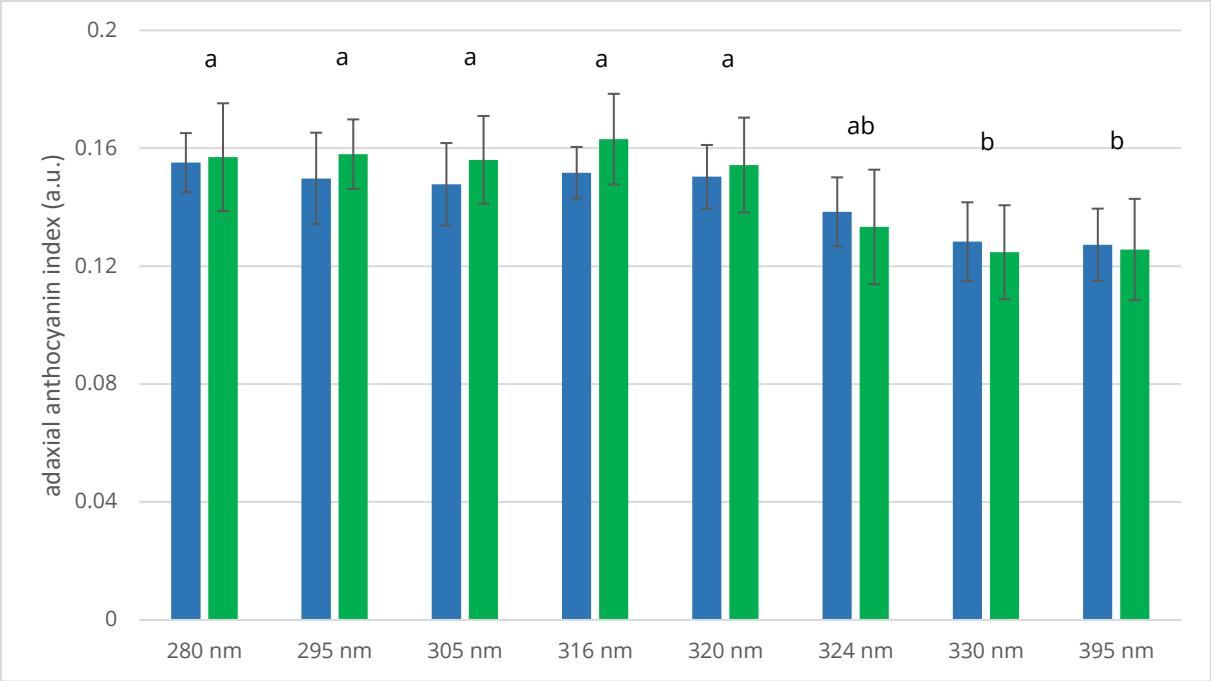

Figure 2. The adaxial anthocyanin index of two *Arabidopsis thaliana* genotypes: Wassilewskija wild-type (Ws) and *uvr8-7*. Column lengths (blue, Ws; green, *uvr8-7*) represent means, and error bars represent standard deviations (n=32). Data were compared by two-way ANOVA and significantly ($p < 0.05$) different means are marked with different letters.

Based on our PSII photochemistry results (well-functioning energy and electron transfer), the decrease in carotenoids or tocopherols more likely refers to a redeployment of biosynthetic resources rather than a stress response. The synthesis of these compounds is descending from the shikimate pathway, and thus it may depend on the amount of materials processed in flavonoid synthesis, which is a dominant response under UV exposure.

On the contrary, UV-B blind *uvr8-7* plants were able to operate their flavonoid synthesis via only the cryptochrome-regulated pathways and thus have had lessened flavonoid synthesis. Therefore, these plants may complement their light protecting ability by strengthening energy dissipating pathways resulting in a higher xanthophyll content. However, the significant decrease in VAZ concentration is suggesting the activation of cryptochrome-related flavonoid biosynthesis under UV transparent filters.

The results of the joint research were presented by the PI at the 4th Network Meeting of UV4Plants, in Krakow, Poland. The poster presentation has been awarded one of the two best poster awards. A manuscript is under construction and will be submitted for publication in a peer-reviewed journal as soon as possible.

Four students (three master's and one bachelor's) were involved in either Arabidopsis or tobacco studies (1 defended, 3 ongoing diploma projects so far). Two of the students presented their data at the local, qualification round of the National Scientific Students' Associations Conference, and both students qualified for the next round.

3. Analysis of UV-acclimation of tobacco genotypes with altered H_2O_2 antioxidant system

Acclimative responses of Wisconsin 38 wild-type tobacco plants developed under natural light conditions were compared to a mutant overexpressing cationic peroxidases (SPI15). Lower chlorophyll content and thus slightly lower PSII quantum yields and electron transport rate were detected in SPI15 leaves, especially under higher PAR intensities. In contrast, higher steady-state cationic POD activity resulted in higher flavonoid and anthocyanin content, which provides more efficient ultraviolet screening and ROS scavenging. The mutation ended in 32% higher activity of superoxide dismutase enzyme (SOD) and thus, elevated hydrogen peroxide production in SPI15 leaves. The equality of total POD activities in the two genotypes suggests the upregulation of alternative H_2O_2 neutralizing pathways (i.e. non-enzymatic antioxidants). Although no difference was detected in POD activity between the two genotypes, the non-denaturing PAGE analysis of leaf samples visualized at least one new POD activity band compared to previous gels representing WT tobacco leaves treated in growth chambers by ultraviolet radiation from an artificial source (Fig 3.).

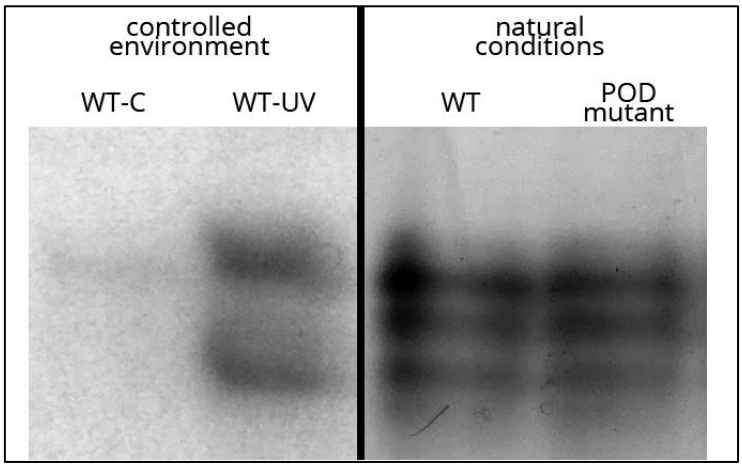

Figure 3. Phenolic peroxidase activities visualized by non-denaturing PAGE using guaiacol staining. On the left, POD bands of WT tobacco leaves are visible. The plants were grown under way lower doses of photosynthetically active radiation than summer daylight (WT-C), treated by supplemental UV radiation from an artificial source (Q-Panel UVB-313EL tubes) (WT-UV). On the right, POD activities of WT and POD mutant tobacco leaves are represented.

4. Triggering ROS homeostasis artificially - a model study on an agricultural plant exposed to ambient UV

In the framework of a collaboration with the Research Institute for Viticulture and Oenology and the Institute of Organic and Medicinal Chemistry, University of Pécs, and the Italian Institute of Technology has been done investigating the possible priming effect of different titanium dioxide (TiO_2) nanoparticles (NPs) on grapevine leaf photochemistry.

TiO_2 NPs are able to induce a UV-driven generation of a variety of reactive oxygen species in the presence of O_2 and H_2O, and this property explains its application in commonly used plant protective materials. However, besides its anti-pathogenic effects, the further consequences of TiO_2 NP applications (ie. on photosynthesis or antioxidant systems) are not been completely explored, yet. So within this collaboration, grapevine (*Vitis vinifera* cv L. Cabernet Sauvignon) leaves have been sprayed by a concentration gradient of rutile and anatase TiO_2 NPs at the Vineyard of University of Pécs during a summer period. The UV exposure of TiO_2 NPs on the leaf surfaces catalyzed the concentration-dependent formation of superoxide or hydroxyl radicals, which affected the PSII quantum efficiency differently. In addition, the application of TiO_2 NPs in appropriate concentrations may lead to elevated organic nitrogen content in leaves. In this study, we determined the TiO_2 concentration threshold which can affect photosynthesis or antioxidant capacities (ie. vitamin B_6 concentration) positively. The active form of vitamin B_6 is an essential building block in the biosynthesis of certain molecules including antioxidant enzymes like superoxide dismutase or phenolic peroxidases. Thus, with the upregulated consumption of inorganic forms of nitrogen, plants may run a strengthened antioxidant system.

The joint work has been published in 2022 in Nanomaterials, a Q1 open access journal.

DISSEMINATION

Partially based on the project's results and experiences, an invited Early Career Researcher review has been published in Journal of Plant Physiology, a D1 journal.

In total, 3 journal publications, 5 conference abstracts, and 1 graduate thesis (supervised by the PI) have been prepared during 3 project years.

Czégény, Gy., Rácz, A. (2023). Phenolic peroxidases: Dull generalists or purposeful specialists in stress responses? Journal of Plant Physiology, 280(14), 153884. https://doi.org/10.1016/j.jplph.2022.153884.

Kőrösi, L., Bognár, B., Czégény, Gy., Lauciello, S. (2022). Phase-selective synthesis of anatase and rutile TiO_2 nanocrystals and their impacts on grapevine leaves: Accumulation of mineral nutrients and triggering the plant defense. Nanomaterials, 12(3), 483. https://doi.org/10.3390/nano12030483

Rácz, A., Czégény, Gy., Csepregi, K., Hideg, É. (2020). Ultraviolet-B acclimation is supported by functionally heterogeneous phenolic peroxidases. Scientific Reports, 10, 16303. https://doi.org/10.1038/s41598-020-73548-5

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