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**Title:**

Elevated ROS-scavenging enzymes contribute to acclimation to UV-B exposure in transplastomic tobacco plants, reducing the role of plastid peroxidases

**Authors:**

Gyula Czégény[1], Bénédicte Le Martret[2], Dóra Pávkovics[1], Philip J. Dix[2], Éva Hideg[1,*]

[1]Department of Plant Biology, Faculty of Sciences, University of Pécs, Pécs, Hungary
[2]Biology Department, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

**\* Corresponding author:**
Prof É. Hideg, Department of Plant Biology, Faculty of Sciences, University of Pécs
postal address: Ifjúság u. 6., Pécs, H-7624, Hungary
tel.: +3672 503600, e-mail address: ehideg@gamma.ttk.pte.hu

**Summary**

Leaf peroxidases play a key role in the successful acclimation of plants to low UV-B doses. The aim of the present study was to examine whether selective enhancement of alternative chloroplast antioxidant pathways achieved by chloroplast transformation affected the need for peroxidase defense. Transplastomic tobacco lines expressing glutathione reductase in combination with either dehydroascorbate reductase or glutathione-S-transferase in their plastids exhibited better tolerance to supplemental UV-B than wild type plants. After 10 days UV treatment, both the maximum and effective quantum yields of PSII decreased in the wild type by 10% but were unaffected in either of the transformed lines. Activities of total peroxidase and ascorbate peroxidase, in addition to dehydroascorbate reductase and gluthatione-S-transferase, were increased by UV in all lines. Gluthatione reductase activity was unaffected by UV in the transplastomic line engineered to have a higher constitutive level of this enzyme, but increased in the two other genotypes. However, the observed more successful acclimation required less activation of peroxidases in the doubly transformed plants than in the wild type and less increase in non-enzymatic hydroxyl radical neutralization in the dehydroascorbate reductase plus glutathione reductase fortified plants than in either of the other lines. These results highlight the fundamental role of efficient glutathione, and especially ascorbate, recycling in the chloroplast in response to exposure of plants to UV-B. They also identify chloroplast localized peroxidases among the large variety of leaf peroxidases as essential elements of defense, supporting our earlier hypothesis on hydrogen peroxide UV-B photo-cleavage as the primary mechanism behind damage.

**Abbreviation**

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); APX, ascorbate peroxidase; AsA, ascorbate; CDNB, 1-chloro-2,4-dinitrobenzene; -C, control plants exposed to PAR only; DG, chloroplast transformant tobacco expressing both DHAR and GR; DHAR,

dehydroascorbate reductase; $F_0$, minimal fluorescence yield of dark-adapted sample with all PS II centers open; $F_m$, maximal fluorescence yield of dark-adapted sample with all PS II centers closed; $F'_m$, maximal fluorescence yield of illuminated sample with all PS II centers closed; $F_v/F_m$, maximum quantum efficiency of PSII; GG, chloroplast transformant tobacco expressing both GR and GST; GR, glutathione reductase; GST, glutathione-S-transferase; HTPA, hydroxyterephthalate; NBT, nitroblue tetrazolium; PAR, photosynthetically active radiation; PH, wild type 'Petit Havana' tobacco; POD, total peroxidase; SOD, superoxide dismutase; TPA, terephthalic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); -UV, plants exposed to PAR and supplemental UV radiation; WT, wild type; $\Phi_{PSII}$, light acclimated effective quantum yield of PSII

**Keywords:**

UV radiation, chloroplast transformant, reactive oxygen species, glutathione reductase, dehydroascorbate reductase, glutathione-S-transferase

**Introduction**

Solar ultraviolet (UV) radiation, especially UV-B (280-315 nm), is known to initiate oxidative damage in leaves, leading to lower photosynthetic activity. Plant responses to UV depend on wavelength, fluence rate, exposure time, and whether plants have been acclimated by prior exposure to UV. For example, plants developing outdoors are usually well-acclimated to solar UV-B, but plants grown indoors under photosynthetically active radiation (PAR, 400-700 nm) only experience the same dose of UV-B as the stressor (Jansen et al. 1998, Hideg et al. 2013). Extreme light stress induced by UV-B may result in DNA, protein and membrane lipid damage. However, even lower doses promote the generation of reactive oxygen species (ROS), either due to metabolic disturbance and impairment of photosynthetic electron transport

or as a result of increased activity of membrane localized NADPH-oxidases and peroxidases (Jenkins, 2009; Hideg et al., 2013, Müller-Xing et al. 2014). Higher $H_2O_2$ concentrations were detected in *Arabidopsis thaliana* leaves in response to a short exposure to 308 nm monochromatic UV (Czégény et al. 2014). In addition, we have shown that peroxidases and efficient hydroxyl radical neutralization are key components of acclimation to supplemental UV radiation (Majer et al. 2014) as these lessen oxidative damage caused by $H_2O_2$ itself or by hydroxyl radicals derived from $H_2O_2$ via UV-B photocleavage (Czégény et al. 2014).

The aim of the present study was to investigate how components of the antioxidant network other than peroxidase contribute to defense against UV in a key cellular compartment, the chloroplast. Previous investigations in our laboratory (Le Martret et al. 2011, Poage et al. 2011) have demonstrated in tobacco that chloroplast transformation (ie. integration and expression of transgenes into the chloroplast genome) can directly increase the activity of several ROS scavenging enzymes in this organelle, resulting in both altered levels and redox states of metabolites (ascorbate and glutathione) and modified response to abiotic stresses. Poage et al. (2011), also showed a diminished impact of UV-B radiation on relative variable fluorescence in transformants expressing either superoxide dismutase (SOD) or glutathione reductase (GR) in their chloroplasts. However, the most pronounced effects of abiotic stress (chilling, salt and methyl viologen-induced oxidative stress) were observed in double transformants (Le Martret et al. 2011) expressing either dehydroascorbate reductase (DHAR) and glutathione reductase (GR), or gluathione reductase and glutathione-S-transferase (GST). These transplastomic plants therefore provide a powerful source for exploring the extent to which chloroplast enzymes contribute to acclimation to UV radiation, and whether reinforcement of ascorbate-glutathione recycling provides an alternative to the direct $H_2O_2$ – $^{\bullet}OH$ neutralizing system. The latter question is relevant to an earlier study of Kubo et al. (1999) who proposed that in *A. thaliana*, environmental stresses may be classified into those which

induce DHAR activity and those which induce ascorbate peroxidase (APX) activity, and classified UV-B in the latter group.

## Materials and methods

*Plant material and UV treatment*

The plants used were *Nicotiana tabacum L.* wild type 'Petit Havana' (PH) and two double plastid transformed lines derived from PH, expressing either dehydroascorbate reductase (from rice) and glutathione reductase (from *E. coli*) (DG), or glutathione reductase and glutathione-S-transferase (both from *E. coli*) (GG). In these plants, foreign DNA is precisely targeted into the same location in the plastome and thus the inter-line variability associated with nuclear transformants can be excluded (Grant et al. 2014) Plants are described in detail in Le Martret et al. (2011). Plants were grown in growth chambers (Sanyo MLR-352H-PE, Panasonic Healthcare Co., Ltd., Oizumi, Japan) at 25/20 °C, at 16 h daily irradiation with ca. 200 $\mu$mol m$^{-2}$ s$^{-1}$ photosynthetically active radiation (PAR). Four-week old plants were divided into two groups each containing 12 plants (4 of each genotype) and were kept for an additional ten days in two separate Fitotrons (SGC 120 Plant Growth Chamber, Weiss Technik UK, Loughborough, UK). The first group (labelled as -UV plants) was kept under 200 $\mu$mol m$^{-2}$ s$^{-1}$ PAR supplemented with UV-B radiation from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) through a cellulose diacetate filter (Courtaulds Chemicals, Derby, UK). The second group (-C, control plants) was kept under 200 $\mu$mol m$^{-2}$ s$^{-1}$ PAR only. The UV radiation was applied to the first group for 4 hours daily, centered at noon. Spectral distribution of UV irradiance was maximal at 318 nm (Majer and Hideg, 2012) and corresponded to 8.8 kJ m$^{-2}$ d$^{-1}$ global (280-400 nm) or 7.7 kJ m$^{-2}$ d$^{-1}$ UV-B (280-315 nm) biologically effective dose, as calculated using the Biological Spectral Weighting Function developed by Flint and Caldwell (2003).

At the end of the ten-day treatment, one fully-developed leaf (node 5) was selected from each plant for analysis. Photosynthetic electron transport was measured on intact leaves on plants, and then the same leaves were frozen in liquid nitrogen and kept at $-20^{o}C$ until used for antioxidant analyses.

*Photosynthetic electron transport measurements*

Chlorophyll fluorescence measurements were carried out using the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). After 30 min dark adaptation, minimum and maximum fluorescence yields $F_o$ and $F_m$ were measured before and after a saturating light pulse, respectively. This was followed by exposure to 55 $\mu$mol m$^{-2}$ s$^{-1}$ blue actinic light for 3 min and light acclimated minimum and maximum fluorescence yields $F'$ and $F'_m$ were obtained using a saturating pulse. Maximal PS II quantum yield was calculated according to Schreiber et al. (1986) as $F_v/F_m = (F_m - F_o) / F_m$ and the light acclimated effective PS II quantum yield ($\Phi_{PSII}$) was characterized as $\Phi_{PSII} = (F'_m - F') / F'_m$ (Genty et al. 1989).

*Sample preparation for antioxidant measurements*

Tobacco leaves were ground in liquid nitrogen into a fine powder, then samples were homogenized in ice cold Na-phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA. When making samples for the ascorbate peroxidase assay 5 mM ascorbate was also included in the grinding buffer. Leaf extracts were centrifuged (24.400 x g, 30 min, 4°C) and supernatants were used for antioxidant capacity measurements. Protein contents were determined according to Bradford (1976).

*Photometric determination of antioxidant enzyme activities*

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All reactions were carried out at room temperature, in 1 mL cuvettes and absorption changes were detected for 90 s using a Shimadzu UV-1800 spectrophotometer in kinetic mode. Straight lines were fitted on absorption vs. time data sets and absorption change per minute slopes were used for calculating enzyme activities with corresponding molar extinction coefficients of substrates.

*Superoxide dismutase* (SOD, EC 1.15.1.1) activity measurement was carried out according to Sun et al. 1988 with slight modifications, based on the inhibition of nitroblue tetrazolium (NBT) reduction by xanthine – xanthine-oxidase generated superoxide anions. 50 mM Potassium phosphate buffer contained the leaf sample, 0.3 mM EDTA, 0.37 mM xanthine and 1.15 mM NBT, and the reaction was started by adding 5 µunit xanthine-oxidase. NBT reduction was followed at 540 nm. Calibration was done with purified SOD (Sigma-Aldrich Kft., Hungary) and enzyme activities were given as µmol SOD min$^{-1}$ g$^{-1}$ protein.

*Total peroxidase* (POD, EC 1.11.1.7) activity was measured via the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method (Childs and Bardsley, 1975) using 10% ABTS, 400 µM $H_2O_2$ in phosphate-citrate buffer (50 mM, pH 5.0). ABTS oxidation was followed at 735 nm. The assay was calibrated with purified horseradish peroxidase enzyme (Sigma-Aldrich Kft., Hungary) and activities in leaf samples were expressed as µmol POD min$^{-1}$ g$^{-1}$protein.

*Ascorbate peroxidase* (APX, EC 1.11.1.11) activities were measured according to Nakano and Asada (1981). The reagent solution contained 0.5 mM ascorbic acid and 1 mM $H_2O_2$ and 1 mM EDTA in a Na-phosphate buffer (50 mM, pH 7.0) and leaf extracts. APX activities were followed as a decrease in absorption at 295 nm as ascorbate was oxidised. Values were corrected for the APX unrelated, direct oxidation of $H_2O_2$ by the added ascorbate and other non-enzymatic antioxidants contained in samples. The applied detection wavelength was slightly higher than the 290 nm used in the original procedure, in order to lessen background

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UV absorption of high leaf polyphenol concentrations present in leaf samples. Accordingly, changes in ascorbate concentrations were calculated using the molar extinction coefficient $\varepsilon_{295nm}$=1.47 mM$^{-1}$ cm$^{-1}$ to express enzyme activities.

*Dehydroascorbate reductase* (DHAR, EC 1.8.5.1) activity was determined by GSH mediated increase in ascorbate absorption at 295 nm according to Hossain and Asada (1984) with the above modification in the applied wavelength. The assay contained 0.25 mM dehydroascorbate and 2 mM GSH in the above buffer and enzyme activities were given as µmol DHAR min$^{-1}$ g$^{-1}$ protein. The reaction rate was corrected for the non-enzymatic direct reduction of dehydroascorbate by GSH, which was below 5% of enzymatic rates typically found in PH-C leaf samples.

*Glutathione S-transferase* (GST, EC 2.5.1.18) activity was assessed using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich Kft., Hungary) as substrate, 5 mM GSH and 1 mM EDTA in 50 mM Na-phosphate buffer (Veal et al. 2002). Enzyme activities were given as nmol GST min$^{-1}$ g$^{-1}$ protein, based on the $\varepsilon_{340nm}$=9.6 mM$^{-1}$ cm$^{-1}$ extinction coefficient of CDNB.

*Glutathione reductase* (GR, EC 1.6.4.2) activity assay was carried out according to Kwon et al. (2003). The reaction was followed as decrease in NADPH absorption at 340 nm for 1 min in a Na-phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA, 1 mM GSSG and 0.2 mM NADPH. Activities as µmol GR min$^{-1}$ g$^{-1}$ protein were calculated using the $\varepsilon_{340nm}$=6.22 mM$^{-1}$ cm$^{-1}$ extinction coefficient of NADPH.

*Determination of hydroxyl radical neutralizing capacity*

For determination of hydroxyl radical scavenging capacity TPA (terephthalic acid) method was used (Šnyrychová and Hideg, 2007), which is based on the fact that antioxidants contained in plant extracts can partly inhibit the oxidation of TPA to hydroxyterephthalate (HTPA) by •OH generated in the assay. Hydroxyl radical antioxidant capacities were

characterized by amounts of plant samples needed to decrease HTPA fluorescence (315 nm excitation, 420 nm emission) by 50% as described earlier (Stoyanova et al. 2011) and were given as µM ethanol equivalent mg$^{-1}$ leaf fresh weight.

*Statistics*

All data are presented as mean averages and standard deviation of the mean. Photosynthetic yield data are averages of four measurements using four leaves from four different plants treated under identical conditions. Photometric assays were done in four repeats with samples from four different plants of the same treatment and genotype. In this way, each data point is an average of 16 values per sample representing both biological variability and technical repetitions. Student's t-test was used to calculate *P*-values, and differences at *P* < 0.05 were considered as significant. All calculations were carried out using Microsoft Excel for Windows (v11.0 2003).

**Results**

In order to test acclimation to UV radiation, both wild type (Petit Havana: PH) and double transplastomic (DHAR plus GR = DG; GR plus GST = GG) tobacco plants (as described by Le Martret et al. 2011) were exposed to daily doses of 7.7 kJ m$^{-2}$ biologically active UV-B radiation. UV supplemented 200 µmol m$^{-2}$ s$^{-1}$ PAR and control plants of each genotype were kept under PAR only. A comparison of leaf photosynthesis at the end of the 10-day treatment showed that UV exposed PH (PH-UV) plants had significantly lower photochemical electron transport than control plants (PH-C). Both maximum efficiency ($F_v/F_m$) and actual quantum yield of PSII electron transport measured in a PAR adapted state ($\Phi_{PSII}$) decreased in PH in response to UV (Table 1.). On the other hand, neither DG-UV nor GG-UV leaves had lower photochemical yields than corresponding controls. In the absence of the UV

treatment, all measured photosynthesis parameters were similar in PH-C, DG-C, and GG-C leaves. Pair wise statistical comparisons of data sets are provided as Supplementary material. These results show that the observed changes were caused by exposure to supplemental UV and suggest that observed UV tolerance of the DG and GG plants relative to PH was due to the antioxidants expressed in them.

As shown in Fig.1., control (not exposed to supplemental UV) transplastomic plants exhibited the expected higher enzyme activities of dehydroascorbate reductase (DHAR), glutathione-S-transferase (GST) and glutathione reductase (GR), compared to those in PH-C. All three enzymes were present at higher activities in UV treated leaves than in corresponding controls. DHAR activities in GG-C leaves were similar to those in PH-C but the enzyme was activated to a larger extent in the GG-UV than in PH-UV samples. Therefore, both DG-UV and GG-UV leaves had higher DHAR activities than those of PH-UV. UV-enhanced DHAR in PH-UV leaves reached the activity which was found in the DHAR expressing plants in the absence of UV treatment (in DG-C). This was not the case with the two other enzymes, GST and GR. The largest, ca 10-fold, increase in GST activity was observed in PH-UV leaves as compared to PH-C, but even this enhanced activity was below the levels in GG-C. DG plants were not reinforced in GST and accordingly, GST activities found in DG-C leaves were not statistically different from those measured in PH-C. However, UV treatment resulted in a smaller, ca. 6-fold, increase in GST activity in DG plants than the 10-fold increase in PH. GR was expressed in both DG and GG plants and enzyme activities in the absence of UV were 52- and 19-times higher in DG-C and in GG-C leaves respectively than in PH-C. UV-induced higher GR activity in PH-UV leaves was significantly lower, than base levels in either transplastomic plant and reached only 4-times the activity found in PH-C. Pair wise statistical comparisons of data sets are provided as Supplementary material.

In addition to enzymes involved in recycling of the two key non-enzymatic antioxidants, AsA and GSH, we also examined how UV affected direct ROS neutralizing pathways. Following the sequence of ROS production via electron transfer $O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH$, SOD, peroxidases and ${}^{\bullet}OH$ neutralizing were measured (Fig.1). In terms of peroxidase defense, there were no significant differences between the two chloroplast transformants, but these plants had distinct antioxidant patterns from wild type plants, during both normal growth and acclimation to UV. Base levels of POD were about half in DG-C and GG-C compared to PH-C, and DG and GG leaves increased their activity in response to UV about 4-times, while this activation was more than 9-fold in PH. On the other hand, both DG-C and GG-C leaves had higher APX and SOD activities than PH-C. Hydroxyl radical neutralizing capacities were not affected by the expression of chloroplast antioxidant enzymes: extracts from GG-C and DG-C leaves were not significantly different from those of PH-C. Capacities increased in all plants in response to UV-B, although to different extents in different genotypes (Fig. 1, see Supplementary material for pair wise comparisons).

## Discussion

Enzyme activities in the transplastomic lines, under PAR only, broadly confirm the increases over wild type reported by Le Martret et al. (2011), with an even greater fold elevation of GR (in DG and GG lines) and GST (in the GG line) than was found previously. Activities of all the engineered enzymes are further induced (in both wild type and transformed lines) by UV-B acclimation, so these enzyme activities remain far higher in the transplastomic lines. The implications of the higher absolute values achieved in the transplastomic lines need to be considered in relation to the perceived roles of these enzymes in ROS amelioration, and the probable interactions between them as they influence the flux of oxidized vs. reduced forms of key antioxidants ascorbate and glutathione. The interplay between these enzymes, the

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antioxidants, and ROS levels is complex and further complicated by the conflicting role of the latter as stressor and signaling molecules (Mittler et al. 2011).

However, some predictions can be made. DHAR and GR catalyse consecutive steps in the ascorbate-glutathione cycle (Mittler 2002). Their dual enhancement should therefore improve the efficiency of regeneration of ascorbate (AsA) as GR reduces glutathione so it remains available as substrate for DHAR. The net effect would be greater availability of AsA and more efficient scavenging of $H_2O_2$ as the whole cycle is stimulated. Elevated levels of AsA in DG lines were reported by Le Martret et al. (2011). The importance of ascorbate recycling in responding to environmental stresses is well established and has been extensively reviewed (Gallie 2013).

GG lines exhibit the predicted increases in GR and GST activity, compared to wild type plants, but this *E. coli* GST enzyme also exhibits glutathione peroxidase (GPX) activity, as has been clearly demonstrated in the GG plants (Le Martret et al. 2011). It is possible that this activity is more important than the GST activity in ROS scavenging as GPX and GR are the two enzymes involved in the glutathione peroxidase cycle, another route for the dissociation of $H_2O_2$. Both pairs of enzymes therefore contribute to cellular protection against abiotic stresses, accounting for the improved tolerance to chilling, salinity and methyl viologen in both the DG and the GG lines (Le Martret et al. 2011). Since both the pathways in which the engineered enzymes are major players involve the neutralization of $H_2O_2$, and neutralization of $H_2O_2$ has been shown to be central to antioxidant defense upon UV-irradiation (Majer et al. 2014), it is reasonable to expect these transformed lines to be altered in their response to UV in comparison to wild type plants.

In interpreting the data on POD and SOD levels in these lines it is reasonable to assume that none of the plants grown under PAR only were subject to strong oxidative stress (as indicated by their high photochemical yields) and thus the observed data are to be explained in

the context of normal metabolism. Higher APX may be necessary to keep cellular $H_2O_2$ concentrations low despite the observed high SOD activities.

In our experiment, peroxidases (both total peroxidase assessed with the synthetic cofactor ABTS, and APX) were activated by the UV treatment in all genotypes. This is in agreement with the results reported by Kubo et al. (1999) using *A. thaliana* leaves and other authors working with several species (Willekens et al. 1994, Yannarelli et al. 2006; Agrawal and Rathore 2007; Berli et al. 2010). In contrast to the observations of Kubo et al. (1999), UV treatment activated both DHAR and APX in our plants. This is less likely to be caused by a difference in the studied species than by a difference in experimental conditions. While the UV irradiation applied by Kubo et al. (1999) resulted in chlorotic spots on leaves after 6 days, plants in our experiment were acclimated to UV, UV exposed leaves had the same chlorophyll content as control ones and only 10% lower steady state photosynthetic electron transport.

Exposure to UV markedly increased ·OH scavenging capacity of the wild type, in accordance with our earlier report (Majer et al. 2014). In the present study we found that DG and GG leaves also increased their ·OH antioxidant capacity, although it was lower in DG-UV and GG-UV than in PH-UV (Fig.1). Due to high reactivity of alcohols to ·OH radicals (Billany et al. 1996), antioxidant capacities can only be assessed using leaf extract made with water-based buffers. Consequently, polyphenols with limited water solubility, such as flavonoids or carotenes are not expected to dominate ·OH antioxidant capacities. Both ascorbate and GSH are abundant water soluble antioxidants which are reactive to ·OH (Foyer and Noctor 2011) and are expected to influence ·OH specific antioxidant capacities of leaf extracts strongly.

In an earlier study using PH tobacco leaves and a supplemental UV treatment which caused 30% decrease in photosynthetic electron transport, we have shown that in UV treated leaves, the extent of activation of ROS neutralizing capacities followed a peroxidases > hydroxyl-radical neutralization > SOD order (Majer et al. 2014). The experiment presented

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here employed a UV source with the same spectral distribution as the previous one. However, during the present work daily UV-B doses were 44% lower and were applied together with an approximately 4-times higher PAR intensity than in the previous experiment. This allowed a better acclimation to UV during the ten days of exposure and consequently PH-UV plants lost only 10% of their photosynthetic electron transport as compared to PH-C. Nevertheless, the extent of antioxidant activation followed a similar order to that in the previous experiment involving a stronger UV stress. As shown in Fig.1, peroxidases were activated to the largest extent and UV-induced increases in ROS scavenging capacities followed a POD (948%) > APX (727%) > $^{\bullet}$OH neutralization (313%) > SOD (151%) order in PH plants.

This was somewhat different in DG and GG plants, which both featured a lower relative increase in POD and APX in response to UV than PH. This may be explained by assuming more effective non-enzymatic neutralization of $H_2O_2$ in the transplastomic plants due to increased recycling of ascorbate and GSH. In DG-UV leaves we measured a POD (448%) > APX (241%) > $^{\bullet}$OH neutralization (185%) ≈ SOD (173%) activation relative to DG-C, suggesting less emphasis on defense against $H_2O_2$ and $^{\bullet}$OH than in PH-UV, and about the same or slightly enhanced protection from $O_2^{\bullet-}$. GG-UV leaves had similar peroxidase activation to that of DG-UV, both being lower than PH-UV. A marked difference between UV-responses of the two chloroplast transformants was in the extent of UV-induced activation of $^{\bullet}$OH neutralization. As opposed to the 185% activation in DG-UV compared to DG-C, GG-UV plants showed much higher, 381% $^{\bullet}$OH neutralization relative to GG-C. A possible explanation is to assume that efficient ascorbate regeneration is more important in defence against $^{\bullet}$OH than GSH recycling, and thus GG-UV leaves needed to activate more $^{\bullet}$OH antioxidant capacity than DG-UV. On the other hand, a comparison of GG-UV and PH-UV leaves illustrates that activation of $^{\bullet}$OH scavenging by itself is not sufficient to protect photosynthesis, as the two genotypes had similar capacities but photochemical yields were affected in PH but not in GG.

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In summary, results presented here show that control of cellular $H_2O_2$ and $^{\bullet}OH$ concentrations is a key factor in successful acclimation to supplemental UV radiation, even in chloroplast transformant plants with enhanced ascorbate and GSH recycling. Both DG and GG plants avoided ROS-induced loss of photosynthetic activity. However, in comparison with wild type plants, these transplastomic lines require less activation of peroxidases (both in DG and GG), and a smaller relative increase in $^{\bullet}OH$ neutralizing capacity (in DG but not in GG), to achieve successful acclimation. The importance and multi-faceted role of ascorbate in UV responses has already been shown under strong oxidative stress conditions achieved by short term high dose UV exposure of ascorbate deficient *Arabidopsis* plants (Gao and Zhang 2008). The novelty of our work lies in showing that while ascorbate is also an important factor under more realistic lower UV-B doses, the key element of long term acclimation to UV-B is enzymatic hydrogen peroxide neutralization in chloroplasts.

## Acknowledgements

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**Figure caption**

**Figure 1.**

Changes in antioxidant enzyme activities and hydroxyl radical scavenging capacities of tobacco leaves in response to UV-B.

PH, 'Petit Havana' wild type tobacco; DG, chloroplast transformant tobacco expressing both DHAR and GR; GG, chloroplast transformant tobacco expressing both GR and GST; -C, control plants exposed to PAR only; -UV, plants exposed to PAR and supplemental UV radiation.

Data are expressed as % of those measured in PH plants kept under PAR only (PH-C plants). Bar lengths correspond to means and error bars represent standard deviations (n=4 for SOD and =16 for all other data). Light grey parts of bars represent values measured in plants kept under PAR only and full bar lengths (light + dark grey) correspond to data measured in UV-acclimated plants (PAR+ UV). In this way, dark grey parts of each bar correspond to UV-induced changes.

With the exception of GR in GG-C and GG-UV, all studied antioxidant capacities were significantly ($P < 0.05$) higher in UV-treated plants than in untreated ones of the same genotype. Asterisks mark data sets where GG-UV or DG-UV plants were different from PH-UV. Pair wise comparisons of all data with statistical analyses and $P$ values are given as Supplementary material.

100% SOD = 480 μmol min$^{-1}$ g$^{-1}$ protein, 100 % POD = 258.8 μmol min$^{-1}$ g$^{-1}$ protein, 100 % APX = 192.8 μmol min$^{-1}$ g$^{-1}$ protein, 100% hydroxyl radical scavenging ($^{\bullet}$OH scav) = 273.52 μM ethanol equivalent g$^{-1}$ fresh leaf weight, 100% DHAR = 4.4 μmol min$^{-1}$ g$^{-1}$ protein, 100% GR = 10.8 μmol min$^{-1}$ g$^{-1}$ protein, 100% GST = 0.654 nmol min$^{-1}$ g$^{-1}$ protein.
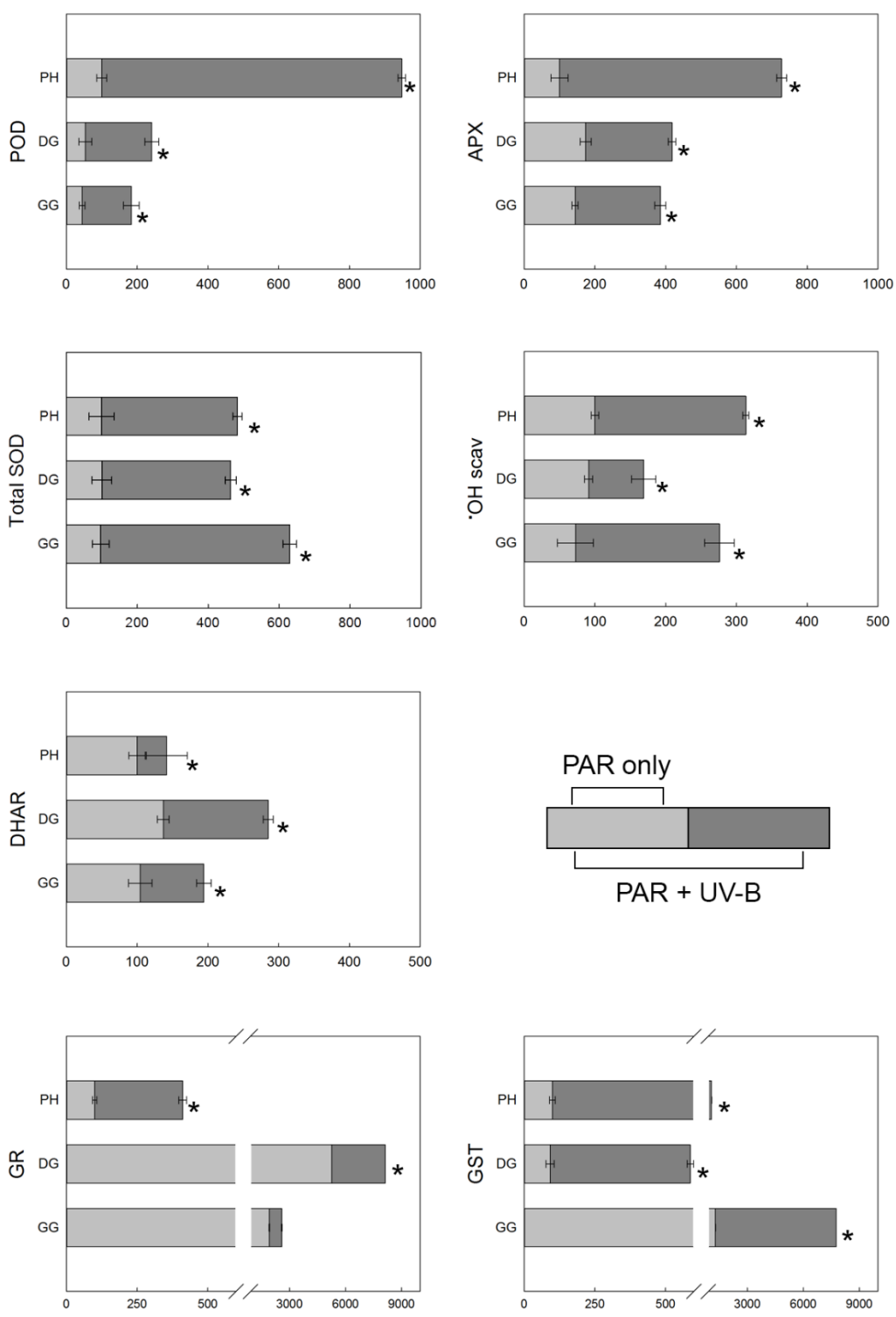
**Table 1:** Effects of supplemental UV radiation on maximum ($F_v/F_m$) and 55 μmol m$^{-2}$ s$^{-1}$ PAR acclimated effective ($\Phi_{PSII}$) quantum yields of PSII.

| Genotype and treatment | $F_v/F_m$ | $\phi_{PSII}$ |
|---|---|---|
| PH-C | 0.854 ± 0.013 | 0.729 ± 0.02 |
| PH-UV | 0.821 ± 0.02 * | 0.663 ± 0.039 * |
| DG-C | 0.849 ± 0.02 | 0.714 ± 0.02 |
| DG-UV | 0.83 ± 0.019 | 0.701 ± 0.027 |
| GG-C | 0.844 ± 0.014 | 0.716 ± 0.014 |
| DG-UV | 0.822 ± 0.017 | 0.698 ± 0.021 |

* Significant difference between control (-C) and UV-exposed (-UV) leaves of the same genotype ($P < 0.05$, n=4)