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## Ultraviolet-B radiation exposure lowers the antioxidant capacity in the *Arabidopsis thaliana* *pdx1.3-1* mutant and leads to glucosinolate biosynthesis alteration in both wild type and mutant†

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Pyridoxine (vitamin B<sub>6</sub>) and its vitamers are used by living organisms both as enzymatic cofactors and as antioxidants. We used *Arabidopsis* pyridoxine biosynthesis mutant *pdx1.3-1* to study the involvement of the PLP-synthase main polypeptide PDX1 in plant responses to ultraviolet radiation of two different qualities, one containing primarily UV-A (315–400 nm) and the other containing both UV-A and UV-B (280–315 nm). The antioxidant capacity and the flavonoid and glucosinolate (GS) profiles were examined. As an indicator of stress,  $F_v/F_m$  of photosystem II reaction centers was used. In *pdx1.3-1*, UV-A + B exposure led to a significant 5% decrease in  $F_v/F_m$  on the last day (day 15), indicating mild stress at this time point. The antioxidant capacity of Col-0 wildtype increased significantly (50–73%) after 1 and 3 days of UV-A + B. Instead, in *pdx1.3-1*, the antioxidant capacity significantly decreased by 44–52% over the same time period, proving the importance of a full complement of functional *PDX1* genes for the detoxification of reactive oxygen species. There were no significant changes in the flavonoid glycoside profile under any light condition. However, the GS profile was significantly altered, both with respect to *Arabidopsis* accession and exposure to UV. The difference in flavonoid and GS profiles reflects that the GS biosynthesis pathway contains at least one pyridoxine-dependent enzyme, whereas no such enzyme is used in flavonoid biosynthesis. Also, there was strong correlation between the antioxidant capacity and the content of some GS compounds. Our results show that vitamin B<sub>6</sub> vitamers, functioning both as antioxidants and co-factors, are of importance for the physiological fitness of plants.

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## Introduction

Vitamin B<sub>6</sub> (VitB<sub>6</sub>) is an essential cofactor involved in over 140 different biochemical reactions.<sup>1</sup> In living cells there are primarily six different VitB<sub>6</sub> analogues, the so called vitamers: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their respective phosphorylated versions (PNP, PLP, and PMP, respectively). Of the phosphorylated VitB<sub>6</sub> species, PLP (pyri-

doxal 5'-phosphate) is the most important, since it is the vitamer that is active as a catalytic cofactor in anabolic and catabolic enzymes. In an *in silico* study, Percudani and Peracchi<sup>2</sup> found that the classes of enzymes that most commonly used PLP as a cofactor were transferases, lyases, isomerases, hydrolases, and oxidoreductases, in descending order of appearance in enzymes that were anticipated to carry this cofactor. In the model plant *Arabidopsis thaliana*, more than 100 genes were assigned to be encoding PLP-binding proteins.<sup>2–5</sup> Mutant studies revealed that decreased VitB<sub>6</sub> synthesis capabilities led to developmental and morphological changes. This included reduced shoot, root, and leaf development,<sup>6–10</sup> and delayed flowering time.<sup>8</sup>

In plants, PLP is synthesized *de novo* from glutamine, ribose 5-phosphate, and glyceraldehyde 3-phosphate by two interacting proteins, the PDX1 PLP synthase and the PDX2 glutaminase.<sup>1,4,11</sup> PDX2 extracts ammonium from glutamine and delivers it to PDX1 which synthesizes the final

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product. PDX1 is a multifunctional enzyme catalysing at least six different chemical reactions. It has been suggested to be one of the most complicated enzymes that exists,<sup>1</sup> with regard to the intricate catalytic processes that are carried out by the polypeptide. The PDX1 PLP synthase exists as a dodecamer *in vivo*<sup>1</sup> and the details of its catalytic function are at present being unveiled.<sup>1,12</sup>

With regard to the physiological role of VitB<sub>6</sub>, increasing scientific evidence shows that VitB<sub>6</sub> also can act as an antioxidant, in addition to its role as a cofactor.<sup>6,13</sup> VitB<sub>6</sub> efficiently detoxifies various reactive oxygen species (ROS) such as singlet oxygen, superoxide anion radical, hydroxyl radical, and hydrogen peroxide.<sup>6,13–19</sup> Thus, in plants, the relatively newly discovered antioxidant activity of VitB<sub>6</sub> has increased the interest of understanding VitB<sub>6</sub> function under different types of environmental stresses.<sup>5</sup> This includes salt stress,<sup>6,7</sup> osmotic stress,<sup>6</sup> photoinhibition,<sup>7,20</sup> and biotic stress and disease resistance.<sup>21,22</sup> Furthermore, VitB<sub>6</sub> has been shown to play an important role in plant responses to ultraviolet-B radiation (UV-B; 280–315 nm), which is a naturally occurring part of the solar spectrum. This role is manifested as an increased expression of VitB<sub>6</sub> biosynthesis genes and enzymes<sup>21,22</sup> and the accumulation of VitB<sub>6</sub> in plants exposed to UV-B.<sup>22</sup> In fact, it is possible that during plant stress in general, VitB<sub>6</sub> could either act as an antioxidant, or as an important cofactor in the stress-protective metabolism, or both. In the *A. thaliana* VitB<sub>6</sub> biosynthesis mutant *pdx1.3-1*, first characterized by Titiz *et al.*,<sup>7</sup> we have previously shown that H<sub>2</sub>O<sub>2</sub> accumulated to a larger extent than in the Col-0 wild type and that the photosynthetic parameters  $F_v/F_m$  (maximal dark-adapted PSII quantum yield) and  $Y(\Pi)$  (effective light-acclimated PSII quantum yield) decreased, whereas the non-photochemical quenching due to dissipative processes ( $Y(\text{NO})$ ) increased.<sup>18</sup> Again, this indicated that the *pdx1.3-1* mutant, lacking full capacity for VitB<sub>6</sub> synthesis, suffered from UV-B-induced oxidative stress. It should be noted that of the three *PDX1* genes, only *PDX1.1* and *PDX1.3* are functional and the *PDX1.3* dominates in the wild type plant.<sup>7</sup> The *pdx1.1pdx1.3* double mutant is embryo lethal.<sup>7</sup>

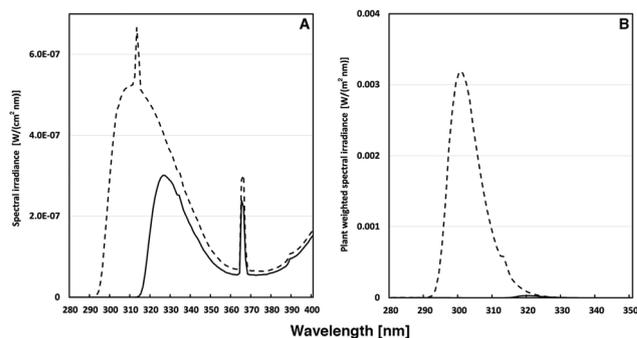
In order to further examine the role of VitB<sub>6</sub> under conditions of supplementary UV-B radiation at levels that generally are not stressful for plants, we devised the present study. Here we further examined the antioxidant capacity of wild type and *pdx1.3-1* plants exposed to supplementary UV-A radiation or UV-A + B radiation, and in plants exposed to photosynthetically active radiation (PAR) only. In addition, we analysed the content and profile of two classes of phytochemicals, flavonoids and glucosinolates (GS). Flavonoids were chosen because their synthesis is a signature UV response in plants<sup>23</sup> and since their biosynthesis is independent of enzymes using VitB<sub>6</sub> as the cofactor. In the case of GS<sup>24–26</sup> they were chosen since one step in the core GS biosynthesis has been confirmed to be dependent on VitB<sub>6</sub>, *i.e.* the one catalysed by the SUR1 S-alkyl-thiohydroximate lyase.<sup>27</sup> Also, three more enzymes involved in the amino acid interconversion leading up to GS biosynthesis have been inferred to use PLP as the cofactor: the

branched-chain-amino-acid aminotransferases 3 (BCAT3;<sup>25</sup> <http://www.uniprot.org/uniprot/Q9M401>), 4 (BCAT4;<sup>28</sup> <http://www.uniprot.org/uniprot/Q9LE06>), and 6 (BCAT6;<sup>29</sup> <http://www.uniprot.org/uniprot/Q9LPM9>), respectively. Thus, with respect to the glucosinolate metabolism, the effects of both the exposure to supplementary UV radiation and the VitB<sub>6</sub> deficiency in the core glucosinolate biosynthesis could be detected.

## Materials and methods

### Materials, growth of plants, light and UV-B exposure conditions

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and the *PDX1.3* T-DNA insertion mutant line *pdx1.3-1* (SALK\_086418) were obtained from The European Arabidopsis Stock Centre, Nottingham, UK (<http://arabidopsis.org>). Seeds were sown in an Arasystem Arabidopsis growth system (Betatech bvba, Ghent, Belgium) on a fertilized compost:perlite (75:25) mixture and incubated in darkness at 4 °C, for 3–5 days. The plants were thereafter grown at 150–190 μmol photons m<sup>-2</sup> s<sup>-1</sup> of PAR using a 16 h light/8 h darkness photoperiod at 22 °C. 40 days after sowing the plants were in addition to the PAR irradiated with UV-A + UV-B (0.25 W m<sup>-2</sup>) or UV-A only (0.11 W m<sup>-2</sup>). The UV-B source (Philips TL40 W/12UV) was covered either with cellulose acetate (UV-A + UV-B) or with Mylar film (UV-A only) to exclude wavelengths shorter than approximately 292 and 315 nm, respectively (Fig. 1A). The cellulose acetate and Mylar filters have minimal influence on the visible light exposure of the plants. The plant-weighted UV-B radiation<sup>30,31</sup> was 36.1 mW m<sup>-2</sup> for the UV-A + B exposed plants and 0.03 mW m<sup>-2</sup> for the UV-A exposed plants (Fig. 1B), corresponding to daily doses of 781 and 1 J m<sup>-2</sup>, respectively. The UV irradiance was measured using an OL754 spectroradiometer (Optronic Laboratories Inc., Orlando, FL). Plants were UV exposed each day for 6 h centered around the solar noon



**Fig. 1** (A) Spectral UV irradiance (in W (cm<sup>2</sup> nm)<sup>-1</sup>) of Philips TL40/12 UV tubes used for the irradiation of *A. thaliana* plants. The UV-A + B light regime (dashed line) was accomplished by filtering the light through cellulose acetate sheets. For the UV-A-enriched light (solid line), Mylar sheets were used as filters. In (B) the corresponding plant-weighted UV (in W (m<sup>2</sup> nm)<sup>-1</sup>)<sup>30</sup> during the two types of exposures is shown.



for 1–15 days. Attached leaves were used for chlorophyll fluorescence. All leaves used for antioxidant and flavonoid and GS phytochemical analysis were harvested after cessation of UV exposure on each indicated day and frozen in liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until freeze drying.

### Chlorophyll fluorescence measurement

The maximal efficiency of PSII ( $F_v/F_m$ )<sup>32</sup> was measured using a Plant Efficiency Analyzer (PEA; Hansatech Instruments, United Kingdom). After 15 min of dark adaptation a minimum of three plants per treatment and at least two of the youngest fully developed leaves from each plant were measured, making the total of measured leaves,  $n = 6\text{--}17$ . One measurement per leaf was performed. The means and standard deviations of these measurements were calculated.

### Antioxidant capacity assay

Hydroxyl radical neutralizing capacities were determined according to Šnyrychová and Hideg.<sup>33</sup> The method is based on the ability of antioxidants contained in leaf extracts to decrease the oxidation of terephthalate (TPA) to hydroxy-terephthalate (HTPA) by hydroxyl radicals generated in the assay. This antioxidant capacity was characterized by the amount of plant sample needed to decrease HTPA fluorescence (315 nm excitation, 420 nm emission) by 50%, as described earlier.<sup>34</sup> The reaction mixture contained 500  $\mu\text{M}$  terephthalate, 100  $\mu\text{M}$  ascorbate, 10  $\mu\text{M}$   $\text{Fe(II)SO}_4$ , and 10  $\mu\text{M}$  EDTA in 50 mM Naphosphate buffer (pH 7.0). The reaction was started by adding 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The assay was calibrated with ethanol, which has high reactivity to hydroxyl radicals, and data were given as mM ethanol equivalent per g leaf dry weight.

### Flavonoid and hydroxycinnamic acid analysis

Flavonoid glycosides and hydroxycinnamic acid derivatives were analyzed according to Schmidt *et al.*<sup>35</sup> with slight modification. Lyophilized, ground plant material (0.02 g) was extracted with 600  $\mu\text{l}$  of 60% aqueous methanol on a magnetic stirrer plate for 40 min at  $20\text{ }^{\circ}\text{C}$ . The extract was centrifuged at 19 000g for 10 min at the same temperature, and the supernatant was collected in a reaction tube. This process was repeated twice with 300  $\mu\text{l}$  of 60% aqueous methanol for 20 and 10 min, respectively; the three corresponding supernatants were combined. The extract was subsequently evaporated until it was dry and was then suspended in 200  $\mu\text{l}$  of 10% aqueous methanol. The extract was centrifuged at 14 500g for 5 min at  $20\text{ }^{\circ}\text{C}$  through a Corning Costar Spin-X plastic centrifuge tube filter (Sigma Aldrich Chemical Co., St Louis, MO) for HPLC analysis. Each extraction was carried out in duplicate.

The composition and content of flavonoid glycosides and hydroxycinnamic acid derivatives were determined from the filtrate using a series 1100 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, binary pump, autosampler, column oven, and photodiode array detector. An Ascentis Express F5 column (150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Supelco) was used to separate the compounds at  $25\text{ }^{\circ}\text{C}$ . Eluent A was 0.5% acetic acid, and eluent B was 100% acetonitrile.

The gradient used for eluent B was 5–12% (0–3 min), 12–25% (3–46 min), 25–90% (46–49.5 min), 90% isocratic (49.5–52 min), 90–5% (52–52.7 min), and 5% isocratic (52.7–59 min). The determination was conducted at a flow rate of 0.85  $\text{ml min}^{-1}$  and a wavelength of 280 nm, 320 nm, 330 nm, 370 nm, and 520 nm. The hydroxycinnamic acid derivatives and glycosides of flavonoids were tentatively identified as deprotonated molecular ions and characteristic mass fragment ions according to Schmidt *et al.*<sup>35</sup> and Neugart *et al.*<sup>36</sup> by HPLC-DAD-ESI-MS<sup>n</sup> using a Bruker amazon SL ion trap mass spectrometer in negative ionisation mode. For the identification of the peaks, the data were compared with the literature of the investigated species and their derivatives. In the mass spectrometer, nitrogen was used as the dry gas (10  $\text{L min}^{-1}$ ,  $325\text{ }^{\circ}\text{C}$ ) and the nebulizer gas (40 psi) with a capillary voltage of  $-3500\text{ V}$ . Helium was used as the collision gas in the ion trap. The mass optimization for the ion optics of the mass spectrometer for quercetin was performed at  $m/z$  301 or arbitrarily at  $m/z$  1000. The MS<sup>n</sup> experiments were performed in auto up to MS<sup>3</sup> in a scan from  $m/z$  200–2000. Standards (chlorogenic acid, quercetin 3-glucoside, kaempferol 3-glucoside and isorhamnetin-3-glucoside; Roth, Karlsruhe, Germany) were used for external calibration curves in a semi-quantitative approach. Results are presented as mg per g dry weight.

### Glucosinolate analysis

The glucosinolate composition of the samples was determined as desulfo-glucosinolates, using a slightly modified method according to Wiesner *et al.*<sup>37</sup> The modifications were as follows: the various desulfo-glucosinolates were separated on a UHPLC-DAD device (UHPLC Agilent 1290 Infinity System, Agilent Technologies, Böblingen, Germany) equipped with a Poroshell 120 EC-C18 column of dimension 100 mm  $\times$  2.1 mm containing particles of size 2.7  $\mu\text{m}$  (Agilent Technologies). The solvent gradient was formed using water (A) and 40% acetonitrile (B), starting at 0.5% B for 2 min, increasing to 49.5% B over the next 10 min, then held for a further 2 min, increased to 99.5% B over the course of 1 min and then held for a final 2 min. The flow rate was 0.4  $\text{mL min}^{-1}$  and the injection volume was 5  $\mu\text{L}$ . Desulfo-glucosinolates were identified by comparing retention times and UV absorption spectra with those of known standards. Quantification was done at 229 nm using the response factor of the GS relative to 2-propenyl glucosinolate (external standard). The determination of glucosinolates was performed in duplicate.

### Statistical analysis

MS Excel was used to calculate the means and standard deviations. Pairwise differences between means were evaluated using two sample Student *t*-tests. The Wizard software (version 1.9.29) for Macintosh was used for this purpose. The number of sampling elements ( $N$ ) in each sample is indicated in corresponding figures. Probabilities of the null hypothesis that the two means are equal ( $p$  values) are indicated in figures as \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.005$ ; and \*\*\*\*  $p < 0.001$ . The com-



parison of treatments (ANOVA) for each phenolic compound was done using Tukey's HSD test at a significance level of 5%.

Correlations between the amounts of glucosinolate compounds and hydroxyl radical neutralizing antioxidant capacities were analysed by calculating Person's correlation coefficient ( $r$ ). Probabilities of the null hypothesis that there is no correlation ( $p$ -values) are shown together with  $r$ -values. A linear connection between hydroxyl radical neutralizing antioxidant capacities and amounts of one of the glucosinolate compounds was studied by calculating a linear fit using the method of least squares. Following this, the probability of the null hypothesis that the slope of a fitting line is equal to zero was examined. The coefficient of determination ( $R^2$ ) was also calculated. These analyses were carried out using PAST software<sup>38</sup> and Statistica™ for Windows™ (version 13.0, Statsoft Inc., Tulsa, OK, USA).

## Results and discussion

While phenolic compounds such as flavonoids and phenolic acids are well known antioxidants in UV-B defense, VitB<sub>6</sub> recently gained attention in the process of cellular oxidative stress.<sup>39</sup> VitB<sub>6</sub> has high antioxidant capacity against hydroxyl radicals<sup>17</sup> and other ROS. Concomitantly, *A. thaliana pdx1.3-1* mutants are highly sensitive to UV-B.<sup>18,20</sup> It has also been shown that the VitB<sub>6</sub> content increased after UV-B but not UV-A exposure in the *A. thaliana* Col-0 wildtype but not in *pdx1.3-1*.<sup>22</sup> However, it has been inferred in a theoretical study that phenolic compounds may be stronger antioxidants than VitB<sub>6</sub>.<sup>40</sup> Indeed, increased flavonoid content in plant leaves due to UV-B exposure is a well-known plant response,<sup>41</sup> whereas the increase in PDX protein levels is comparably small.<sup>41</sup> On the other hand, UV-A radiation did not have any major effect on the flavonoid abundance of Arabidopsis leaves.<sup>41</sup>

Therefore, we devised the present experimental set-up to study the role of VitB<sub>6</sub> vitamers in the UV-B-regulation of flavonoid and GS profiles on the one hand, and the antioxidant capacity of leaves of the Col-0 and *pdx1.3-1 A. thaliana* accessions, on the other. As a proxy for distress,<sup>42</sup> we also measured the dark-adapted maximal photosynthetic capacity in the form of the chlorophyll fluorescence parameter  $F_v/F_m$ . This parameter has previously been shown to differ between the two Arabidopsis varieties after a short, high dose UV exposure.<sup>18</sup> Thus, the use of low dose exposure as in the present study is novel.

### Maximum quantum yield of PSII photochemistry, $F_v/F_m$

$F_v/F_m$  was measured in *A. thaliana* plants exposed to control light (PAR only), UV-A- or UV-A + B-supplemented PAR. The exposures were carried out for 0, 1, 2, 3, 4, 8, or 15 days, six hours per day centered around the solar noon. No significant differences were found as a function of genotype *per se*. Before day 15, significant decreases in  $F_v/F_m$  compared with the corresponding controls were only found on day 3 and 4 for the *pdx1.3-1* mutant illuminated with supplementary UV-A + B (Fig. 2; Table 1). This may reflect insufficient protection due to decreased antioxidant capacity in *pdx1.3-1* leaves (see below).

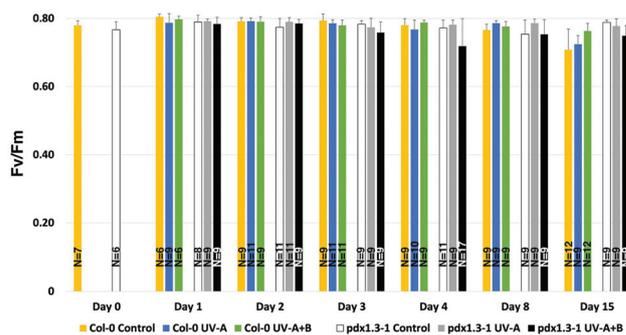


Fig. 2 Maximum quantum yield of PSII photochemistry ( $F_v/F_m$ ) was measured in *A. thaliana* plants exposed to control light, UV-A- or UV-A + B-supplemented light, given for 0, 1, 2, 3, 4, 8, or 15 days, six hours per day centered around the solar noon. The bars indicate the standard deviation. Significant changes between pairwise physiologically relevant comparisons of samples are shown in Table 1 and means and standard deviations in Table S1.†  $N = 6$ –17 leaves from at least three different plants were measured.

Interestingly, changes in  $F_v/F_m$  were seen also after 15 days, although this was not the case for the 8-day exposures. In fact, there was a small but significant difference in  $F_v/F_m$  in Col-0 on day 15 between the control and UV-A-treated plants on the one hand, and the UV-A + B-treated plants on the other (by 0.039–0.055 units). Also, there was a significantly lower  $F_v/F_m$  in the Col-0 control plants than in the *pdx1.3-1* controls after 15 days of exposure (by 0.080 units). Lowering of the  $F_v/F_m$  in Col-0 on day 15 may be attributed to the first signs of bolting that had occurred in all nine plants in this accession at that time-point but only in one of the nine *pdx1.3-1* plants (not shown). Thus, the *pdx1.3-1* mutation may lead to delayed bolting compared with the wild type. This may in turn be due to a lower abundance in *pdx1.3-1* of metabolites needed for the transition from the vegetative to the reproductive stage.

However, this finding is in contrast to the case in the *A. thaliana pdx3* mutant.<sup>43</sup> The *pdx3* mutant is deficient in the gene encoding the PDX3 PMP/PNP oxidase of the VitB<sub>6</sub> salvage pathway that functions to interconvert the vitamers PMP and PNP to PLP. The *pdx3* mutant instead displayed an early flowering phenotype that was linked to the over-accumulation of PMP and a concomitant impaired nitrogen metabolism.<sup>43</sup> In addition, bolting in itself may considerably alter the metabolism of Arabidopsis plants, *e.g.* in the form of increased oxidative pressure as a result of decreased ascorbate peroxidase and catalase activities.<sup>44,45</sup>

Taken together, these results indicate that the plants had not been subjected to any distress<sup>42</sup> caused by the UV treatments. However, eustress may have occurred in the *pdx1.3-1* plants exposed to UV-A + B, particularly after 3 days of exposure or longer.

### The hydroxyl radical neutralizing capacity

The antioxidant capacity of mature (40-day old) Col-0 wild type and *pdx1.3-1* mutant plants was measured. This was done



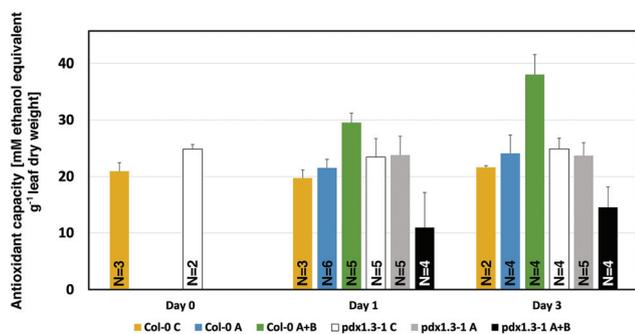
**Table 1** Significant changes in  $F_v/F_m$  (Fig. 1) between pairwise physiologically relevant comparisons of samples carried out using Student's  $t$ -test. Symbols denote: \*  $0.05 > p > 0.01$ , \*\*  $0.01 > p > 0.005$ , \*\*\*  $0.005 > p > 0.001$ , \*\*\*\*  $p < 0.001$

Day	Genotype	Treatment	Significance	Day	Genotype	Treatment
0	Col-0	Control	***	1	Col-0	Control
0	Col-0	Control	**	15	Col-0	Control
3	<i>pdx1.3-1</i>	Control	*	3	<i>pdx1.3-1</i>	UV-A + B
4	Col-0	UV-A	*	4	Col-0	UV-A + B
4	<i>pdx1.3-1</i>	Control	*	4	<i>pdx1.3-1</i>	UV-A + B
4	<i>pdx1.3-1</i>	UV-A	*	4	<i>pdx1.3-1</i>	UV-A + B
8	Col-0	Control	*	8	Col-0	UV-A
8	Col-0	Control	*	15	Col-0	Control
8	Col-0	UV-A	****	15	Col-0	UV-A
8	<i>pdx1.3-1</i>	Control	*	8	<i>pdx1.3-1</i>	UV-A
8	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	Control
8	<i>pdx1.3-1</i>	UV-A	*	8	<i>pdx1.3-1</i>	UV-A + B
15	Col-0	Control	**	15	Col-0	UV-A + B
15	Col-0	Control	****	15	<i>pdx1.3-1</i>	Control
15	Col-0	UV-A	***	15	Col-0	UV-A + B
15	<i>pdx1.3-1</i>	Control	****	15	<i>pdx1.3-1</i>	UV-A + B
15	<i>pdx1.3-1</i>	UV-A	*	15	<i>pdx1.3-1</i>	UV-A + B

3 hours after the solar noon on the day before the onset of UV-A or UV-A + B exposure (day 0), or at 3 hours after the solar noon on the first and third day of UV exposure (day 1 and day

3, respectively). As is evident from Fig. 3 and Table 2, UV-A exposure did not give rise to any changes in this antioxidant capacity, neither after 1 nor 3 days of exposure, in any of the two *Arabidopsis* accessions, compared with the controls. However, there was a strongly significant increase in antioxidant capacity in Col-0 already after exposure to supplementary UV-A + B radiation for one day. An equally significant decrease in antioxidant capacity in *pdx1.3-1* indicates the lack of induction of any protective response against the highly oxidizing hydroxyl radical in UV-B exposed plants when only one functional *PDX1* gene was present.

Interestingly, in the *pdx1.3-1* mutant the  $\cdot\text{OH}$  neutralizing antioxidant capacity of the control plants (day 0, day 1, and day 3; Fig. 3), is higher than in the corresponding controls by 15–19%. This is similar to the case in another *PDX1 Arabidopsis* mutant (*rsr4-1*) used in UV-B exposure studies.<sup>19</sup> The reason for this higher background antioxidant capacity in *rsr4-1* was attributed to the contribution of  $\cdot\text{OH}$  scavenging by other non-enzymatic antioxidants. Particularly chlorogenic acid (a plant phenolic compound) and  $\alpha$ -tocopherol were considered the main candidates for this.<sup>19</sup>



**Fig. 3** Hydroxyl radical antioxidant capacity measurement in *A. thaliana* Col-0 wildtype and *pdx1.3-1* mutant plants 0, 1, and 3 days (6 hours per day around the solar noon) after supplementary UV-A or UV-A + B exposure. The bars indicate the standard deviation. Significant changes between pairwise physiologically relevant comparisons of samples are shown in Table 2.  $N = 2$ –6 replicates per sample type.

**Table 2** Significant changes in the antioxidant capacity of samples as shown in Fig. 3. Pairwise physiologically relevant comparisons of samples were carried out using Student's  $t$ -test. Symbols denote: \*  $0.05 > p > 0.01$ , \*\*  $0.01 > p > 0.005$ , \*\*\*  $0.005 > p > 0.001$ , \*\*\*\*  $p < 0.001$

Day	Genotype	Treatment	Significance	Day	Genotype	Treatment
0	Col-0	Control	*	0	<i>pdx1.3-1</i>	control
1	Col-0	Control	****	1	Col-0	UV-A + B
1	Col-0	Control	*	1	<i>pdx1.3-1</i>	control
1	Col-0	UV-A	****	1	Col-0	UV-A + B
1	Col-0	UV-A + B	***	3	Col-0	UV-A + B
1	Col-0	UV-A + B	****	1	<i>pdx1.3-1</i>	UV-A + B
1	<i>pdx1.3-1</i>	Control	**	1	<i>pdx1.3-1</i>	UV-A + B
1	<i>pdx1.3-1</i>	UV-A	**	1	<i>pdx1.3-1</i>	UV-A + B
3	Col-0	Control	***	3	Col-0	UV-A + B
3	Col-0	UV-A	****	3	Col-0	UV-A + B
3	Col-0	UV-A + B	****	3	<i>pdx1.3-1</i>	UV-A + B
3	<i>pdx1.3-1</i>	Control	***	3	<i>pdx1.3-1</i>	UV-A + B
3	<i>pdx1.3-1</i>	UV-A	***	3	<i>pdx1.3-1</i>	UV-A + B



**Table 3** Flavonol glycosides (mg per g dry weight) measured in *A. thaliana* plants exposed to control light, UV-A- or UV-A + B-supplemented light, for 2, 3, 4, 8, or 15 days, six hours per day centered around the solar noon. Values with the same letters are not significantly different (Tukey's HSD test is calculated at  $p \leq 0.05$ ) for each flavonol glycoside. Capital letters represent the ANOVA for the effect of UV radiation for each day and genotype. There were no significant differences between the genotypes. Q: quercetin; K: kaempferol; rha: rhamnose; glc: glucose; rut: rutinoides

Days of exposure	Treatment	Genotype	Q-3-rha-7-glc	K-3-rha-7-rha	K-3-glc-7-rha	K-3-rut-7-glc	Average: total flavonoids			
2	Control	Col-0	5.10 ± 3.84	7.49 ± 0.97	1.94 ± 2.73	5.24 ± 0.19	B	22.98 ± 2.89	B	
	UV-A	Col-0	1.79 ± 1.16	5.07 ± 0.13	1.84 ± 1.03	2.85 ± 0.06	AB	15.31 ± 1.45	AB	
	UV-A + B	Col-0	0.53 ± 0.43	4.06 ± 3.14	1.97 ± 1.62	2.25 ± 1.73	A	9.69 ± 7.23	A	
	Control	<i>pdx1.3-1</i>	3.51 ± 5.01	8.50 ± 0.35	B	5.18 ± 1.37	B	6.34 ± 0.61	B	
	UV-A	<i>pdx1.3-1</i>	0.62 ± 0.24	4.73 ± 0.50	A	2.66 ± 0.47	A	2.45 ± 0.15	A	
	UV-A + B	<i>pdx1.3-1</i>	0.89 ± 0.13	4.93 ± 1.60	A	1.98 ± 0.82	A	2.58 ± 0.60	A	
4	Control	Col-0	0.15 ± 0.02	8.54 ± 0.83	AB	1.26 ± 0.19	4.82 ± 0.22	18.85 ± 2.43	AB	
	UV-A	Col-0	0.19 ± 0.05	5.69 ± 2.90	A	0.94 ± 0.51	3.25 ± 1.87	13.01 ± 6.33	A	
	UV-A + B	Col-0	0.28 ± 0.12	11.56 ± 0.69	B	1.28 ± 1.15	7.22 ± 3.32	25.55 ± 4.73	B	
	Control	<i>pdx1.3-1</i>	0.49 ± 0.43	4.96 ± 1.00	A	1.67 ± 0.82	A	2.90 ± 0.13	A	
	UV-A	<i>pdx1.3-1</i>	0.53 ± 0.57	4.64 ± 1.49	A	0.97 ± 0.72	A	2.68 ± 0.63	A	
	UV-A + B	<i>pdx1.3-1</i>	0.35 ± 0.30	10.49 ± 0.72	B	5.02 ± 1.08	B	7.35 ± 0.23	B	
8	Control	Col-0	0.13 ± 0.01	3.71 ± 1.16	2.05 ± 0.47	2.25 ± 0.64	8.56 ± 2.19	A		
	UV-A	Col-0	0.33 ± 0.26	4.69 ± 2.15	1.30 ± 0.44	2.61 ± 1.24	10.00 ± 3.956	A		
	UV-A + B	Col-0	0.12 ± 0.01	8.42 ± 2.81	4.43 ± 3.38	6.56 ± 2.83	21.34 ± 4.11	B		
	Control	<i>pdx1.3-1</i>	0.13 ± 0.01	2.94 ± 0.52	1.40 ± 0.33	1.66 ± 0.29	6.51 ± 1.13	A		
	UV-A	<i>pdx1.3-1</i>	0.32 ± 0.04	3.42 ± 1.73	3.42 ± 1.13	1.98 ± 0.93	10.60 ± 1.02	A		
	UV-A + B	<i>pdx1.3-1</i>	0.43 ± 0.29	7.50 ± 4.87	1.33 ± 1.36	9.47 ± 2.46	22.31 ± 3.17	B		
15	Control	Col-0	0.29 ± 0.03	4.53 ± 0.83	A	1.93 ± 0.42	2.71 ± 0.48	A	11.25 ± 1.71	A
	UV-A	Col-0	0.34 ± 0.27	4.69 ± 1.30	A	2.12 ± 0.63	2.57 ± 1.75	A	10.94 ± 3.68	A
	UV-A + B	Col-0	0.24 ± 0.11	10.99 ± 0.48	B	6.15 ± 4.96	8.61 ± 2.93	B	26.44 ± 8.43	B
	Control	<i>pdx1.3-1</i>	0.48 ± 0.31	3.81 ± 0.51	A	0.90 ± 0.46	2.26 ± 0.15	A	8.30 ± 0.70	A
	UV-A	<i>pdx1.3-1</i>	0.44 ± 0.22	4.54 ± 0.68	A	1.70 ± 0.18	2.66 ± 0.54	A	10.78 ± 0.61	A
	UV-A + B	<i>pdx1.3-1</i>	0.29 ± 0.07	10.72 ± 0.83	B	3.64 ± 4.57	9.77 ± 0.73	B	24.96 ± 6.02	B

**Table 4** Sinapic acid derivatives (mg per g dry weight) measured in *A. thaliana* plants exposed to control light, UV-A- or UV-A + B-supplemented light for 2, 3, 4, 8, or 15 days, six hours per day centered around the solar noon. Values with the same letters are not significantly different (Tukey's HSD test calculated at  $p \leq 0.05$ ) for each sinapic acid derivative. Capital letters represent the ANOVA for the effect of UV radiation for each day and genotype. Lowercase letters represent the ANOVA for the effect of plant age (days). There were no significant differences between the genotypes

Days of exposure	Treatment	Genotype	Sinapoyl-glucoside	Sinapoyl-malate	Sinapic acid	Average: total sinapic acid derivatives					
2	Control	Col-0	0.11 ± 0.06	a	0.99 ± 0.75	ab	0.51 ± 0.04	b	1.61 ± 0.72	b	
	UV-A	Col-0	0.18 ± 0.03		0.74 ± 0.34		0.96 ± 0.91		1.88 ± 0.64		
	UV-A + B	Col-0	0.12 ± 0.10		0.10 ± 0.03		0.30 ± 0.20		0.52 ± 0.31		
	Control	<i>pdx1.3-1</i>	0.12 ± 0.05		2.69 ± 1.63	B	1.03 ± 1.53		3.83 ± 1.39	B	
	UV-A	<i>pdx1.3-1</i>	0.24 ± 0.25		0.43 ± 0.09	AB	1.18 ± 0.93		1.85 ± 0.61	AB	
	UV-A + B	<i>pdx1.3-1</i>	0.08 ± 0.00		0.17 ± 0.07	A	0.78 ± 0.43		1.03 ± 0.50	A	
4	Control	Col-0	0.88 ± 0.48	b	0.93 ± 0.14	A	b	0.23 ± 0.04	a	2.04 ± 0.66	b
	UV-A	Col-0	0.46 ± 0.47		0.60 ± 0.56	A		0.41 ± 0.48		1.47 ± 0.84	
	UV-A + B	Col-0	0.32 ± 0.31		2.20 ± 0.27	B		0.08 ± 0.01		2.60 ± 0.12	
	Control	<i>pdx1.3-1</i>	0.37 ± 0.48		0.50 ± 0.23			0.14 ± 0.10		1.01 ± 0.81	
	UV-A	<i>pdx1.3-1</i>	0.19 ± 0.19		0.60 ± 0.70			0.57 ± 0.46		1.35 ± 0.36	
	UV-A + B	<i>pdx1.3-1</i>	0.73 ± 0.57		1.34 ± 1.11			0.11 ± 0.05		2.18 ± 0.82	
8	Control	Col-0	0.06 ± 0.00	a	0.06 ± 0.00	a	a	0.09 ± 0.01	a	0.21 ± 0.01	a
	UV-A	Col-0	0.11 ± 0.07		0.14 ± 0.03			0.29 ± 0.29		0.54 ± 0.35	
	UV-A + B	Col-0	0.07 ± 0.05		0.77 ± 0.57			0.07 ± 0.00		0.90 ± 0.56	
	Control	<i>pdx1.3-1</i>	0.06 ± 0.00		0.06 ± 0.00			0.07 ± 0.00		0.19 ± 0.00	
	UV-A	<i>pdx1.3-1</i>	0.06 ± 0.00		0.29 ± 0.12			0.64 ± 0.90		0.99 ± 0.94	
	UV-A + B	<i>pdx1.3-1</i>	0.08 ± 0.03		1.07 ± 1.06			0.64 ± 0.43		1.79 ± 1.23	
15	Control	Col-0	0.34 ± 0.24	a	0.21 ± 0.23	a	a	0.35 ± 0.29	a	0.90 ± 0.26	B
	UV-A	Col-0	0.24 ± 0.16		0.09 ± 0.01			0.29 ± 0.03		0.61 ± 0.16	AB
	UV-A + B	Col-0	0.07 ± 0.00		0.09 ± 0.04			0.06 ± 0.00		0.22 ± 0.04	A
	Control	<i>pdx1.3-1</i>	0.06 ± 0.00	A	0.22 ± 0.13			0.14 ± 0.06	AB	0.42 ± 0.07	
	UV-A	<i>pdx1.3-1</i>	0.06 ± 0.00	A	0.17 ± 0.05			0.49 ± 0.28	B	0.72 ± 0.32	
	UV-A + B	<i>pdx1.3-1</i>	0.07 ± 0.00	B	0.13 ± 0.03			0.07 ± 0.01	A	0.27 ± 0.03	



## Flavonoids and sinapic acid

The analysis of the flavonoid glycosides and hydroxycinnamic acid derivatives in leaves of *Arabidopsis* Col-0 and the *pdx1.3-1* mutant revealed a highly dynamic picture with regard to the total content and the relative abundance of each of the chemical species over 15 days of UV exposure (Tables 3 and 4). In general, sinapic acid and its derivatives were more prominent constituents in younger than in older leaves. The total levels of flavonoids remained constant over time.

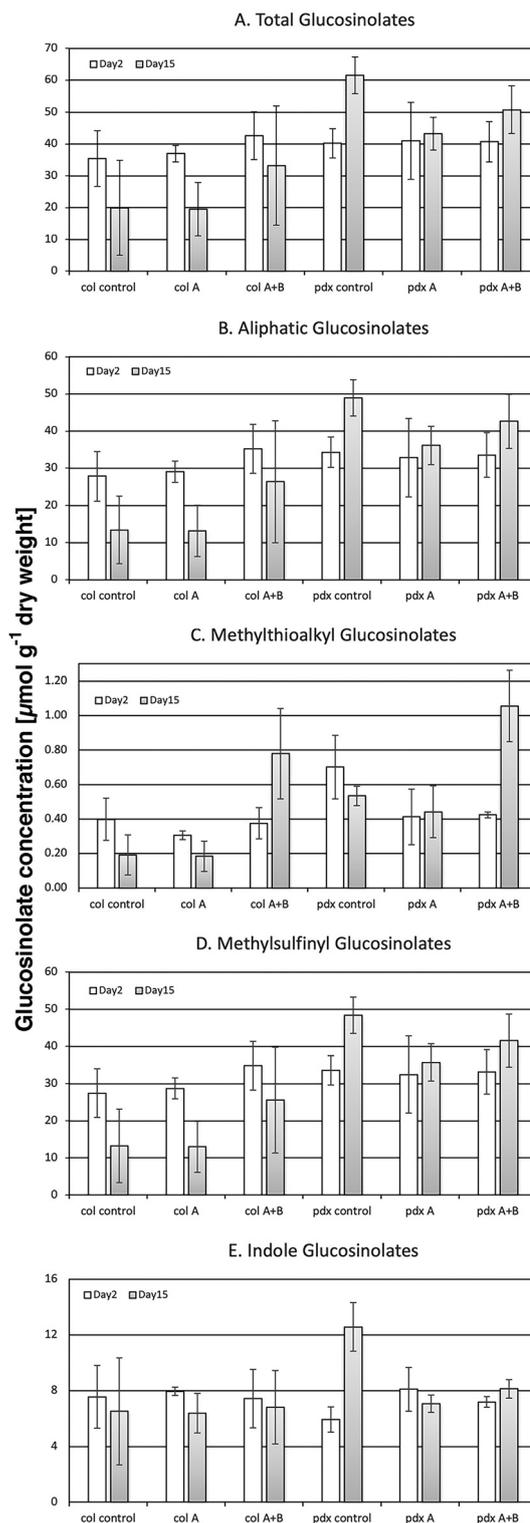
The major sinapic acid derivative sinapoyl-malate increased after 4 days of UV-A + B exposure in Col-0 whereas sinapoyl glucoside increased after 15 days of UV-A + B exposure in *pdx1.3-1* (Table 4). Thus, our data indicate that sinapic acid derivatives do not contribute to any large extent in *Arabidopsis* UV defense. In fact, as shown by Heinze *et al.*,<sup>46</sup> these compounds decrease in abundance with plant maturation and only partly vary with UV irradiation during growth. Although an increased content of hydroxycinnamic acid derivatives has been suggested to be an important response to UV exposure in *A. thaliana*,<sup>47</sup> we have generally found these compounds to be less important than flavonoid glycosides.<sup>48</sup>

For flavonoid glycosides, there was a general shift in total flavonoid content between day 2 and the later days of the study. In the 2-day time point, the total flavonoid levels in both *Arabidopsis* accessions were higher in the non-UV controls. In the later time-points the UV-A + B-exposed plants always had a higher content of flavonoids in leaves than both the controls and the UV-A-exposed plants (Table 3). This is reminiscent of the situation in kale (*Brassica oleracea* var. *sabellica*),<sup>49</sup> where the quercetin glycoside content decreased after one day of UV-B treatment and increased in the following days of UV-B treatment, concomitantly with the mRNAs encoding flavonol 3'-hydroxylases. The effect of the additional UV-B in the present study was substantial after 4 days and further on.

The difference in the total flavonoid glycoside content and flavonoid glycoside profile between the Col-0 wild type and the *pdx1.3-1* mutant was in principle non-existent at all time-points when the same exposure conditions were compared. This underscores the lack of interaction between flavonoid biosynthesis and VitB<sub>6</sub>.

With regard to the individual flavonoid glycosides, quercetin-3-rhamnoside-7-glucoside was present to a relatively large extent in both *Arabidopsis* accessions on day 2 in the non-UV control but to a considerably smaller extent after exposure to both UV-based light regimens (Table 3). While Demkura *et al.*<sup>49</sup> and Götz *et al.*<sup>50</sup> found an increase of quercetin glycosides in *Arabidopsis* after UV-B exposure, our results are again in line with those obtained with kale.<sup>51</sup> From day 4 and onwards, the quercetin-glycoside was hardly detectable in any of the samples.

UV-A and UV-A + B exposure led to an initial (day 2) decrease of kaempferol glycosides in *pdx1.3-1*. UV-A + B exposure then resulted in an increase of these compounds on day 4 in *pdx1.3-1* leaves. Kaempferol-3-rhamnoside-7-rhamnoside and kaempferol-3-rutinoside-7-glucoside contents



**Fig. 4** Content (in  $\mu\text{mol per g dry weight}$ ) of total (A), aliphatic (B), methylthioalkyl (C), methylsulfinylalkyl (D) and indole glucosinolates (E) in *Arabidopsis thaliana* Col-0 wild type and the *pdx1.3-1* mutant exposed to control light, or UV-A- or UV-A + B-supplemented light after 2 and 15 d ( $n = 3$ ); the bars indicate the standard deviation. Significant changes between pairwise physiologically relevant comparisons of samples are shown in Table 5.



increased in Col-0 and *pdx1.3-1* leaves of UV-exposed plants on day 15. Consequently, *pdx1.3-1* shows a more extended and faster response to UV. The results support the hypothesis of VitB<sub>6</sub> being an antioxidant that can detoxify various ROS.<sup>15,17–19</sup> Indeed, the increased content of kaempferol glycosides has previously been shown to be part of the UV response in several *Brassicaceae*<sup>51–53</sup> and in *Arabidopsis*.<sup>49</sup>

### Glucosinolates

Ten individual glucosinolates were quantitatively analyzed in *A. thaliana* Col-0 wild type and its mutant *pdx1.3-1*. In both genotypes the aliphatic glucosinolates represented the majority (by content and by the number of chemical species) of glucosinolates. Particularly the subgroup of methylsulfinylalkyl glucosinolates (3-methylsulfinylpropyl, 4-methylsulfinylbutyl, 5-methylsulfinylpentyl, 7-methylsulfinyl-heptyl, and 8-methylsulfinyloctyl) dominated, primarily in the form of the 4-methylsulfinylbutyl glucosinolate. Precursors of the methylsulfinylalkyl glucosinolates, *i.e.* methylthioalkyl glucosinolates, were also found, but only as minor components of the total GS. Only two of the five methylsulfinylalkyl glucosino-

late precursors were present: 3-methyl-thiopropyl and 4-methylthiobutyl glucosinolates. The group of indole glucosinolates was comprised of the indole 3-indolylmethyl glucosinolate and its derivatives 4-hydroxy-3-indolylmethyl and 1-hydroxy-3-indolylmethyl glucosinolates. However, the latter compounds were only present in trace amounts.

The two genotypes could be differentiated by their corresponding individual glucosinolate levels. The *pdx1.3-1* mutant (*pdx* control, in Fig. 4) showed a constitutively higher total glucosinolate content than the Col-0 wild type (*col* control, in Fig. 4), particularly and significantly with ongoing ontogeny at day 15 (Fig. 4A and Table 5). This significant genotype effect on day 15 was also reflected in the glucosinolate subgroups (aliphatic, methylthioalkyl, methylsulfinylalkyl and indole GS; Fig. 4B–E and Table 5). The higher GS levels in the *pdx1.3-1* mutant were thus present although there was a reduced availability of VitB<sub>6</sub> due to the knock-out of one of the two functional *PDX1* genes. This may seem surprising since VitB<sub>6</sub> is necessary as a cofactor in GS synthesis. PLP is present in BCAT3, BCAT4 and BCAT6 enzymes, and active in the formation of the methionine side-chain elongation as a precursor

**Table 5** Significant changes in the glucosinolate (GS) content of samples as shown in Fig. 4. Pairwise physiologically relevant comparisons of samples were carried out using Student's *t* test. Symbols denote: \* 0.05 > *p* > 0.01, \*\* 0.01 > *p* > 0.005, \*\*\* 0.005 > *p* > 0.001. A–E is according to Fig. 4

Day	Genotype	Treatment	Significance	Day	Genotype	Treatment
<b>A</b>						
<b>Total GS</b>						
2	Col-0	UV-A	*	15	Col-0	UV-A
2	<i>pdx1.3-1</i>	Control	**	15	<i>pdx1.3-1</i>	Control
15	Col-0	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	UV-A	*	15	<i>pdx1.3-1</i>	UV-A
15	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	UV-A
<b>B</b>						
<b>Aliphatic GS</b>						
2	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	UV-A	**	15	<i>pdx1.3-1</i>	UV-A
15	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	UV-A
<b>C</b>						
<b>Methyl thioalkyl GS</b>						
2	<i>pdx1.3-1</i>	UV-A + B	**	15	<i>pdx1.3-1</i>	UV-A + B
15	Col-0	Control	*	15	Col-0	UV-A + B
15	Col-0	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	UV-A	*	15	Col-0	UV-A + B
15	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	UV-A + B
15	<i>pdx1.3-1</i>	UV-A	*	15	<i>pdx1.3-1</i>	UV-A + B
<b>D</b>						
<b>Methyl sulfinyl GS</b>						
2	Col-0	UV-A	*	15	Col-0	UV-A
2	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	Control	*	15	<i>pdx1.3-1</i>	Control
15	Col-0	UV-A	*	15	<i>pdx1.3-1</i>	UV-A
15	Col-0	UV-A + B	*	15	<i>pdx1.3-1</i>	UV-A
<b>E</b>						
<b>Indole GSs</b>						
2	<i>pdx1.3-1</i>	Control	***	15	<i>pdx1.3-1</i>	Control
15	<i>pdx1.3-1</i>	Control	**	15	<i>pdx1.3-1</i>	UV-A
15	<i>pdx1.3-1</i>	Control	*	15	<i>pdx1.3-1</i>	UV-A + B



of aliphatic glucosinolates.<sup>29</sup> It is also necessary for the activity of SUR1,<sup>27</sup> which is an enzyme synthesizing thiohydroxamic acid that in turn is glycosylated and sulfonated to form the core structure of all glucosinolates. However, in our present study, VitB<sub>6</sub> is not limiting the glucosinolate formation in *pdx1.3-1* and has a higher glucosinolate content than Col-0.

In contrast to the case with the flavonoids, the levels of all glucosinolates in the *pdx1.3-1* mutant were significantly reduced under one or both UV treatments, most obvious after extended UV-A treatment on day 15 (Fig. 4B, D, E and Table 5). Although the glucosinolate profile changed differently to UV exposure with respect to plant species,<sup>54</sup> Wang *et al.*<sup>55</sup> also reported a decrease in the concentration of total glucosinolates in *A. thaliana* after 12 h of UV-B treatment (1.55 W m<sup>-2</sup>). However, in our present study, using considerably lower UV levels (0.7 kJ m<sup>-2</sup> d<sup>-1</sup> of plant weighted UV-B), nearly all glucosinolates in the Col-0 wild type were unaffected by both UV treatments used (Fig. 4B, D, E and Table 5). Furthermore, Demkura<sup>49</sup> showed no effect of UV-B (5.5 kJ m<sup>-2</sup> d<sup>-1</sup> of plant weighted UV-B) on GS. This lack of UV response indicates that glucosinolates have no primary function in response to UV (especially UV-B) in the way flavonoids have.

Notwithstanding, in both *Arabidopsis* genotypes, the minor methylthioalkyl glucosinolates were exceptions to the lack of UV response, which is reported here for the first time. On day 15 of exposure, they showed a distinct and statistically significant increase under UV-A + B radiation compared with the corresponding controls and compared with exposures to UV-A only (Fig. 4C and Table 5). This response was primarily determined by the accumulation of the 4-methylthiobutyl glucosinolate, which is a precursor in 4-methylsulfinylbutyl glucosinolate formation. Thus, this effect suggests that under the 15-day UV-A + B exposure, there was a negative impact on the 4-methylthiobutyl to 4-methylsulfinylbutyl side chain oxidation carried out by the flavin monooxygenase FMO GS-OX5 (an enzyme that does not use VitB<sub>6</sub> as a co-factor). No such impact was seen after UV-A exposure (Fig. 4C and Table 5). These results were in contrast to a UV study using a shorter UV-A + B exposure at a lower dose.<sup>56</sup> In that study, UV-A + B-treated broccoli sprouts (0.3 kJ m<sup>-2</sup> d<sup>-1</sup> given for 5 days) exhibited an increased methylsulfinylbutyl glucosinolate content. This increase was matched with a corresponding up-regulation of the *FMO GS-OX5* gene. Thus, this suggests that there are both UV-related and genetic differences between the two plant species – *Arabidopsis* vs. broccoli – with regard to the conversion of 4-methylthiobutyl to 4-methylsulfinylbutyl glucosinolate.

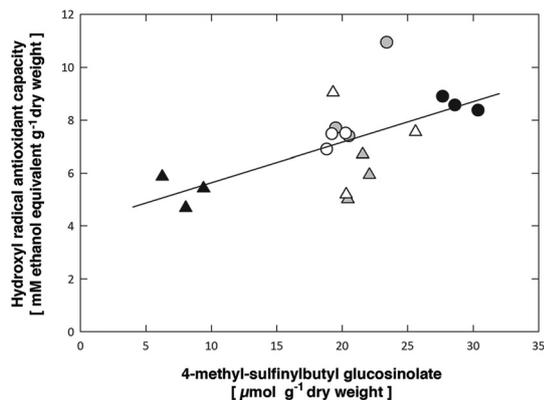
Particularly the content of the precursor 4-methylthiobutyl glucosinolate was correlated with a suppression of the antioxidant capacity (Tables 6 & S2<sup>†</sup>). On the other hand, the content of the more abundant short-chain methylsulfinylalkyl glucosinolates (3-methylsulfinylpropyl, 4-methylsulfinylbutyl) was positively correlated with the hydroxyl radical antioxidant capacity in both *Arabidopsis* accessions. An increased formation of ROS, *e.g.* under UV-A + B exposure, can be assumed to result in an increased formation of ROS scavenging metab-

**Table 6** Significance of pairwise differences (*p*-values; above diagonal) and correlation analysis (correlation coefficients, Pearson's *r*; below diagonal). Significant positive differences are shown in bold and significant negative differences in italic. Marginally positive differences are labelled using a bold italic font

Compound	aox-OHrad	3-m-spr	4-m-sbut	5-m-spe	3-m-tp	4-m-tbut	7-m-shep	i-3-m	8-m-soct	4-m-3-im	1-m-3-im	7-m-thep	8-met-toct	Total GS
aox-OHrad														
3-m-spr	0.5583													
4-m-sbut	0.6113	0.9539												
5-m-spe	0.0669	-0.0398	0.2401											
3-m-tp	0.2870	0.2211	0.4508	0.8047										
4-m-tbut	-0.4990	-0.6127	-0.5087	0.3253	0.2731									
7-m-shep	0.4504	0.5426	0.7189	0.6610	0.8895	0.0536								
i-3-m	0.5030	0.8812	0.8899	0.1449	0.2756	-0.5199	0.5874							
8-m-soct	0.0160	-0.1077	0.0959	0.6714	0.7852	0.7334	0.6662	-0.0689						
4-m-3-im	0.4799	0.4747	0.6344	0.6153	0.4959	-0.3927	0.5041	0.5532	0.0658					
1-m-3-im	0.4026	0.5861	0.6597	0.2499	0.2549	-0.3641	0.4300	0.7929	-0.0203	0.4837				
7-m-thep	-0.2390	-0.1425	-0.0523	0.3359	0.4297	0.7248	0.3598	0.0587	0.6613	-0.1204	0.1044			
8-met-toct	-0.1689	-0.0132	0.1044	0.4119	0.4858	0.6099	0.4469	0.2121	0.6392	0.0369	0.2974	0.9462		
Total GS	0.5747	0.9119	0.9842	0.3416	0.5333	-0.3932	0.7880	0.9141	0.2063	0.6497	0.7007	0.0911	0.2460	

The following abbreviations were used: aox-OHrad, hydroxyl radical antioxidant capacity; 3-m-spr, 3-methyl-sulfinylpropyl; 4-m-sbut, 4-methyl-sulfinylbutyl; 5-m-spe, 5-methyl-sulfinylpentyl; 3-m-tp, 3-methyl-thiobutyl; 4-m-tbut, 4-methyl-thiobutyl; 7-m-shep, 7-methyl-sulfinylheptyl; i-3-m, indolyl-3-methyl; 8-m-soct, 8-methyl-sulfinyloctyl; 4-m-3-im, 4-methoxy-3-indolylmethyl; 1-m-3-im, 1-methoxy-3-indolylmethyl; 7-m-thep, 7-methyl-thioheptyl; 8-met-toct total, 8-methyl-thiooctyl; GS, total glucosinolate content.





**Fig. 5** Significant ( $p = 0.00702$ ) positive correlation between the leaf 4-methyl-sulfinylbutyl glucosinolate content and the hydroxyl radical antioxidant capacities. Symbols show data for *Arabidopsis thaliana* Col-0 (circles) or *pdx1.3-1* mutant leaves (triangles) exposed to control (open), UV-A-supplemented (grey) or UV-A + B-supplemented (black) light. The solid line shows the linear regression,  $R^2 = 0.373$  (see Table 6 for the statistical analysis and Table S3† for ordinary least squares regression fit of data).

olites, such as the short-chain methylsulfinylalkyl glucosinolates (Fig. 5), in contrast to the UV-A treatment, which is not expected to lead to ROS formation. However, the decreased conversion of 4-methylthiobutyl to 4-methylsulfinylbutyl glucosinolate prevents the contribution of the latter compound to the antioxidant capacity in both Col-0 and *pdx1.3-1* under UV-A + B treatment. Moreover, in the UV-A-exposed plants there was neither an accumulation of 4-methylthiobutyl glucosinolate nor an increase in 4-methylsulfinylbutyl glucosinolate. This suggests that the antioxidative potential of the short-chain methylsulfinylalkyl glucosinolates is not utilized in these *Arabidopsis* accessions. Accordingly, Taviano *et al.*<sup>57</sup> proposed that glucosinolates are not directly involved in the primary antioxidant activity. Instead, these authors suggested that the ferrous iron-chelating properties of glucosinolates protect cells from oxidative stress caused by ROS.

## Conclusions

Our experiments using two different UV light regimens in exposure studies with Col-0 wild type *Arabidopsis* and *A. thaliana* plants deficient in VitB<sub>6</sub> biosynthesis (*pdx1.3-1*) led to the following conclusions:

1. The use of 15 days of UV-A or UV-A + B exposure with *Arabidopsis* Col-0 did not lead to any distress, as judged by  $F_v/F_m$  chlorophyll fluorescence levels, that were only marginally affected by the exposures. In the *pdx1.3-1* mutant, UV-A + B exposure led to a significant 5% decrease in  $F_v/F_m$  on day 15 of the experiment, indicating the development of eustress.
2. The antioxidant capacity of Col-0 increased after 1 and 3 days of UV-A + B exposure by at least 50%. In the *pdx1.3-1* mutant the antioxidant capacity decreased by at least 40% over the same time period.

3. No significant changes in flavonoid content and profile between *Arabidopsis* accessions were found. Starting from day 4, UV-A + B treatments led to the highest content of total flavonoid glycosides.

4. The glucosinolate profile was significantly changed, both with regard to the *Arabidopsis* accession and the UV exposure, during the exposure period. This most likely reflects the presence of at least one VitB<sub>6</sub> vitamers cofactor-dependent enzyme in the GS biosynthesis pathway.

5. The antioxidant capacity of the *Arabidopsis* genotypes and their GS profiles were correlated: short-chain methylsulfinylalkyl GS were positively correlated with the antioxidant capacity in both Col-0 and *pdx1.3-1*, whereas the content of 4-methylthiobutyl GS was correlated with suppression of the antioxidant capacity.

Our results thus confirm the important and dual role of VitB<sub>6</sub> as both an antioxidant and an enzymatic co-factor in plants.

## Conflicts of interest

There are no conflicts to declare.

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