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Research highlights

► UV-B induces increased levels of pyridoxine and the PDX1 protein in *Arabidopsis*. ► The isoenzyme to be increased is PDX1.1. ► PDX1 is located in mesophyll and pallisade parenchyma but not in epidermis. ► In the *pdx1* mutant, the UV-B-induced increase in pyridoxine is inhibited. ► High and medium dose UV-B-signalling pathways are partially inhibited in the mutant.

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Research article

The role of the pyridoxine (vitamin B_6) biosynthesis enzyme PDX1 in ultraviolet-B radiation responses in plants

Mikael Ristilä [a], Hilja Strid [b], Leif A. Eriksson [a,c], Åke Strid [a,*], Helena Sävenstrand [a]

[a] School of Science and Technology and Örebro Life Science Centre, Örebro University, SE-70182 Örebro, Sweden
[b] School of Medicine and Health Sciences and Örebro Life Science Centre, Örebro University, SE-70182 Örebro, Sweden
[c] School of Chemistry, National University of Ireland, Galway, Galway, Ireland

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ABSTRACT

Ultraviolet-B radiation regulates plant growth and morphology at low and ambient fluence rates but can severely impact on plants at higher doses. Some plant UV-B responses are related to the formation of reactive oxygen species (ROS) and pyridoxine (vitamin B_6) has been reported to be a quencher of ROS. UV-B irradiation of *Arabidopsis* Col-0 plants resulted in increased levels of PDX1 protein, compared with UV-A-exposed plants. This was shown by immunoblot analysis using specific polyclonal antibodies raised against the recombinant PDX1.3 protein and confirmed by mass spectrometry analysis of immunoprecipitated PDX1. The protein was located mainly in the cytosol and also in the membrane fraction of plant leaves. Immunohistochemical analysis performed in pea revealed that PDX1 is present in UV-B-exposed leaf mesophyll and palisade parenchyma but not in epidermal cells. Pyridoxine production increased in Col-0 plants exposed to 3 days of UV-B, whereas in an *Arabidopsis pdx1.3* mutant UV-B did not induce pyridoxine biosynthesis. In gene expression studies performed after UV-B exposure, the *pdx1.3* mutant showed elevated transcript levels for the *LHCB1*3* gene (encoding a chlorophyll *a*/*b*-binding protein of the photosystem II light-harvesting antenna complex) and the pathogenesis-related protein 5 (*PR-5*) gene, compared with wild type.

1. Introduction

Sunlight is essential for the survival of any photosynthetic organism, both as a source of energy and as a source of information about e.g. diurnal rhythms. In addition to the wavelengths in the visible, the influx of solar radiation also contains ultraviolet-B radiation (UV-B; 280–315 nm), among other spectral regions. Ambient UV-B radiation has important regulatory effects on plant growth and morphology, whereas high levels of UV-B are stressful. The deleterious effects include DNA damage [1], damage to the photosynthetic apparatus [2], and alteration of the structural integrity of membranes [3]. As one response to the perception of UV-B radiation, plants show altered expression of genes encoding components of different protective pathways, such as detoxification of reactive oxygen species [4], and production of UV-B-absorbing pigments [5,6]. In addition, increased expression of pathogenesis-related genes, such as *PR-5* [7], occurs. Also, decreased expression of different photosynthetic genes [8,9] is the result of lowered chloroplast activities.

In addition, a large number of other genes regulated by low level UV-B radiation have been identified [10,11]. One of the more interesting genes up-regulated by UV-B was a pyridoxine/pyridoxal 5-phosphate (vitamin B_6) biosynthesis gene, *AtPDX1.3* (denoted *PyroA* in Brosché et al.) [10]. *AtPDX1* is a highly conserved gene, and homologues are found in a variety of eukaryotes and in some species of bacteria. Pyridoxine and its derivatives are synthesized by microorganisms and plants and are essential co-factors of a number of metabolic enzymes, including enzymes that catalyze transaminations, decarboxylations, and racemations of amino acids. Pyridoxine is also an efficient quencher of various reactive oxygen species (ROS) such as singlet oxygen and superoxide [12,13]. In studies using *Arabidopsis* knock-out mutants, it has been shown that pyridoxine is required for tolerance to salt and osmotic stress [12]. Additionally, pyridoxine seems to be required for post-embryonic root development [12].

The *de novo* biosynthesis of pyridoxine follows two distinct pathways, which do not coexist in any organism [13,14]. In one of the two pathways, the one used by *Escherichia coli* and some other eubacteria, pyridoxine is synthesized from 1-deoxy-D-xylulose-5-phosphate and

* Corresponding author. Tel.: +46 19 303603; fax: +46 19 303566.
E-mail address: ake.strid@oru.se (Å. Strid).

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4-phosphohydroxy-L-threonine by the pyridoxine synthase proteins PdxA and PdxJ [15]. In the alternative pyridoxine biosynthesis pathway, used by e.g. yeast, fungi, and plants, the general understanding is that pyridoxal-5-phosphate is synthesized from glutamine, ribose 5-phosphate (or ribulose 5-phosphate), and glyceraldehyde 3-phosphate (or dihydroxyacetone phosphate) by two proteins, PDX1 and PDX2, which function in a complex as a glutamine amidotransferase [16,17]. PDX2 possesses glutaminase activity. Thus it extracts an ammonium group from glutamine that is incorporated into the product [18], and PDX1 accepts this ammonium group and synthesizes the final product [16,17]. PDX2 requires PDX1 to show glutaminase activity and therefore forms a complex with this polypeptide [16,18]. When determining the crystal structure of PdxS (a PDX1 homologue from *Geobacillus stearothermophilus*), Zhu et al. [19] concluded that PDX1 exists as a dodecamer *in vivo*.

Based on the completely sequenced genome of *Arabidopsis thaliana*, three homologues of *AtPDX1* have been identified, *PDX1.1–1.3*. Tambasco-Studart et al. [17] have shown that only the first and the third of these are functional homologues. Additionally, Wagner et al. [20] have shown that both *PDX1.1* and *PDX1.3* can form a functional complex with the enzymatic partner *PDX2*. *PDX1.2*, on the other hand, could not. Based on alignment of the translated protein sequences, the two polypeptides PDX1.1 and PDX1.3 show an identity of 87%. For *PDX2*, encoding the second pyridoxal 5-phosphate biosynthesis gene, only a single homologue has been identified in the *Arabidopsis* genome. This gene was shown to be essential for plant viability since the *pdx2* knock-out mutant was arrested in embryo development [17].

Since ultraviolet-B radiation can give rise to ROS and the *PDX1.3* gene was shown to be up-regulated by UV-B radiation [10], it was of interest to study further the role of PDX1 in UV-B-exposed plants. We investigated the regulation of the level of PDX1 in UV-B-irradiated wild type *Arabidopsis* and the localisation of the protein in leaves, by using specific antibodies. The PDX1 isozyme involved in the UV-B response was determined and *Arabidopsis* knock-out mutants were used to study both the gene expression pattern of UV-B marker genes and to examine the accumulation of pyridoxine under UV-B treatment. The results show that PDX1/pyridoxine is indeed involved in plant UV-B responses.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana ecotype Columbia-0 (Col-0) was used. The *pdx1.3* T-DNA mutant lines *pdx1.3-1* and *pdx1.3-2* (SALK_086418 and SALK_129277) were obtained from The European Arabidopsis Stock Centre (http://arabidopsis.info). Seeds were grown on fertilized soil:vermiculite:perlite mixture and incubated in darkness at 4 °C, for 4 days. The plants were thereafter grown at 100 μmol photons m^{-2} s^{-1}, 16 h photoperiod, 22 °C day/night, 65% relative humidity.

Soaked *Pisum sativum* seeds were planted in vermiculite and grown in 22 °C, 16 h light and 8 h darkness. The plants were watered with Hoagland solution [21].

2.2. UV-B treatment

4 weeks after sowing the plants were in addition to visible light irradiated with UV-A + UV-B (0.81 W m^{-2}) or UV-A only as a control (0.31 W m^{-2}). 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. The biologically effective UV-B radiation was normalized to 300 nm ($UV\text{-}B_{BE,300}$ = 0.18 W m^{-2}) according to Caldwell [22] and Green et al. [23] The UV radiation was measured with an OL754 UV–vis Spectroradiometer (Optronic Laboratories Inc., Orlando, FL). Plants were exposed either for 0, 3 and 9 h of radiation and used for RNA isolation and Northern blotting, or for 6 h of radiation each day (between 9 a.m. and 3 p.m.) for 1–7 days and used for protein extraction, western blotting, immunoprecipitation, and pyridoxine determinations. All leaves in a rosette were harvested and frozen in liquid nitrogen and kept at −80 °C until further use. The experiments were repeated at least three times.

For immunohistochemistry, 20-day-old pea plants were exposed to UV-A + UV-B (0.87 W m^{-2}; $UV\text{-}B_{BE,300}$ = 0.3 W m^{-2}) radiation during 5 days (6 h of radiation each day; 9 a.m.–3 p.m.). Control plants were exposed to UV-A (0.64 W m^{-2}) radiation only during the same time periods.

2.3. RNA blot analysis

Total RNA was isolated from rosettes according to the method described by Carpenter and Simon [24]. 10 μg of total RNA was separated on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane, Hybond-N (GE Healthcare, Uppsala, Sweden), by pressure blotting. Labeling of probes and hybridization were performed according to Sävenstrand et al. [25]. Control of RNA loading and transfer was accomplished by hybridization with a radiolabelled 18S ribosomal cDNA probe [6] to the same membrane after stripping. The intensity of the blots was quantified with ChemiDoc XRS (Bio-Rad Laboratories CA, USA). The following molecular markers were used: *CHS* (At5g13930, *AtCHS* cDNA a gift from Dr Frederick M. Ausubel, Harvard Medical School), *LCHB1*3* (At1g29930, obtained from the Arabidopsis Biological Resource Center, Ohio State University, clone pAB140; Ref. [26]), *MEB5.2* (At3g17800, Ref. [10]), and *PR-5* (At1g75040, the cDNA a gift from Novartis AG, Research Triangle Park, NC).

2.4. Protein extractions

To extract the cytosolic protein fraction, approximately 200 mg of irradiated leaves was crushed and transferred to an Eppendorf tube. 100 μl of Tris–HCl (50 mM, pH 8) was added and homogenized using a plastic pestle (VWR International, Stockholm, Sweden). The samples were centrifuged twice at 10 000 rpm for 10 min and the soluble fraction was used for western blotting. The amount of protein in the extracts was determined using the Bradford protein assay (Bio-Rad, Hercules, CA), according to the manufacturer instructions.

The membrane protein fraction was extracted from 1 g of irradiated leaves. 1 ml cold grinding buffer (0.4 M sorbitol; 20 mM Tricine–NaOH, pH 7.8; 20 mM NaCl; 5 mM $MgCl_2$; 250 μM PMSF) was added to the leaves and kept on ice. The samples were homogenized with an Ultra Turrax (IKA Labortechnik, Staufen, Germany) by two 30 s pulses at 13 000 rpm. The homogenates were filtered through 4 layers of miracloth (Calbiochem Corp, La Jolla, CA), and the filtrates were transferred to Eppendorf tubes. After centrifugation at 2900*g* for 5 min at 4 °C, the pellets were homogenized in 1 ml cold wash buffer (10 mM Tricine–NaOH, pH 7.8; 10 mM NaCl; 10 mM $MgCl_2$). After removing a 20 μl sample for Chl determination [27], the remainder of the homogenates were centrifuged at 8000*g* for 5 min at 4 °C and the pellets were resuspended in storage buffer (7 mM Tris–base, pH 7.8; 53 mM glycine; 10% glycerol) to a final concentration of 1.1 mg Chl ml^{-1} and then quickly frozen in liquid N_2.

2.5. Cloning of the PDX1 gene into the pET8c expression vector

The open reading frame of *PDX1.3*, corresponding to the intron-free gene At5g01410, was amplified from genomic Wassilewskija

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(Ws) wild type DNA by PCR. The primers used (DNA Technology A/S, Aarhus, Denmark) introduced an XhoI and an MluI restriction enzyme site at the 5′-end and the 3′-end of the resulting cDNA, respectively. After restriction, the open reading frame was cloned into the pET8C expression vector. The vector adds a His_6-tag at the N-terminus of the recombinant protein and is under control of the T7 promoter. The construction was verified by sequencing using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA).

2.6. Purification of recombinant protein and antibody production

E. coli BL21(DE3)Star cells containing the *PDX1.3*-pET8C construct were grown in LB-medium containing 2.5 mM betaine (Sigma–Aldrich Corp, St Louis, MO) and 100 μg ml^{-1} ampicillin at 37 °C. Protein expression was induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM, when the OD_{600} had reached 0.6. Five hours after induction, the culture was harvested and pellets were quickly frozen in liquid nitrogen and stored at −80 °C until further use.

The cells were broken using an X-press (AB Biox, Göteborg, Sweden) and resuspended in 50 mM NaP_i, pH 8.0; 200 mM NaCl; 5 mM L-histidine; 0.05% Tween-20; 1 mM PMSF and sonicated on an ice bath for 10 min. The suspension was centrifuged at 45 000 rpm for 45 min at 4 °C (Beckman 70Ti rotor). The supernatant was mixed with Ni–NTA resin (Sigma–Aldrich) and incubated on rotating rolls for 1 h at 4 °C. The sample was transferred to a chromatography column and washed with the same buffer as used above. The recombinant PDX1.3 protein was eluted using the same buffer supplemented with 150 mM L-histidine. After diluting the eluate 100-fold with 10 mM Tris–HCl, pH 8.0; 10% glycerol, it was further purified using anion exchange chromatography with the ÄKTA system (GE Healthcare, Uppsala, Sweden) and a ResourceQ-column. The recombinant protein was eluted with a gradient of 0–1.0 M NaCl in 10 mM Tris–HCl, pH 8.0; 10% glycerol. Pooled fractions were concentrated, and the buffer changed to 10 mM Tris–HCl, pH 8.0; 10% glycerol using an Amicon Ultra-15 centrifugal filter, 30 kDa MW cut off (Millipore, Billerica, MA). The Bradford protein assay was used to determine the protein concentration in the sample according to the manufacturer instructions (Bio-Rad Inc). For antibody production, either native or SDS-denatured protein was injected into rabbits. Immunisation, maintenance of rabbits, and collection of serum were carried out by Davids Biotechnologie (Regensburg, Germany).

2.7. Enzymatic assay

The pyridoxal 5-phosphate synthetic activity was measured according to Tambasco-Studart et al. [17]. Briefly, the reaction mixture contained 50 mM Tris–HCl, pH 8; 500 μM ribose 5-phosphate (Sigma–Aldrich); 1 mM glyceraldehyde 5-phosphate (Sigma–Aldrich); 10 mM ammonium sulphate (Sigma–Aldrich) and 1.33 mg ml^{-1} of isolated protein. The build up of pyridoxal 5-phosphate (PLP) was observed by recording the increase in absorbance at 414 nm in a Shimadzu UV1601 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan). The reactions were carried out at 37 °C. The extinction coefficient ($\varepsilon_{PLP,414nm}$) was empirically determined to 5643 $M^{-1}\,cm^{-1}$ by measuring the absorbance at 414 nm in a series of different PLP concentrations in the above-mentioned reaction mixture, omitting the isolated protein.

2.8. Western blotting

Protein from leaf extracts (12 μg per lane for the cytosolic fraction or 3.3 and 5.5 μg Chl for the membrane fractions, respectively) or from recombinant protein samples was separated using SDS–PAGE 12% precast gels (Ready-Gel, Bio-Rad, Hercules, CA) and the protein was transferred onto Hybond-ECL nitrocellulose membrane (GE Healthcare, Uppsala, Sweden) by electroblotting. After blocking for 1 h at room temperature in TBS-T buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.1% (v/v) Tween-20) containing 5% (w/v) non-fat dry milk, the membrane was incubated for 1 h at room temperature in primary antibody diluted with TBS-T buffer containing 5% non-fat dry milk. The membrane was washed 2 times 5 min in TBS-T buffer before adding the secondary antibodies, Anti-Rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI), diluted with TBS-T containing 5% non-fat dry milk. After incubating the membrane for 1 h at room temperature, the washing step was repeated. The antibody binding bands were visualized by incubating the membrane in AP buffer (100 mM Tris–HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer recommendations (Promega, Madison, WI).

2.9. Immunoprecipitation

PDX1.3 antibodies were purified from rabbit serum by affinity chromatography. 4 mg of PDX1.3 protein was coupled to CnBr-activated sepharose 4B gel (Pharmacia, Uppsala, Sweden) and packed into a column. 5 ml of PDX1.3 antiserum was loaded to the column and after washing, the purified antibody was eluted with 5 ml 50 mM glycine buffer (pH 2.5; 0.1% Triton X-100; 0.15 M NaCl) directly into 1 M of Tris–HCl pH 9.0. The purified antibody was desalted and concentrated using an Amicon Ultra-15 centrifugal filter, 10 kDa MW cut off (Millipore, Billerica, MA) and stored in 100 μl of PBS buffer. 15 μl of purified antibody was bound to the AminoLink plus Coupling Resin and loaded into a column (Pierce® direct IP kit, Pierce Biotechnology, USA) according to the manufacturer instructions. 250 μl of ice-cold IP buffer was added to 250 μl of *Arabidopsis* leaf material that had been exposed to UV-B irradiation for 3 days. The leaves were crushed together with the buffer and incubated on ice for 5 min. The solution was centrifuged for 10 min at 13 000 rpm and the supernatant was loaded onto the IP column and incubated at 4 °C over night. The column was washed repeatedly according to the manual and the protein eluted directly into 1 M Tris–HCl (pH 9.0). 24 μl of precipitated protein was loaded onto an SDS–Page gel and the Coomassie Brilliant Blue-stained band was sent for MS analysis (Alphalyse, Odense, Denmark).

2.10. Immunohistochemistry

Pea tissue was positioned in cassettes and fixed in Histofix (effective osmolarity 300 mOsmol; Histolab Products AB, Göteborg, Sweden) for 6–24 h. The tissue was dehydrated through a graded series of ethanol (70%, 90%, 95%) to xylene (30%, 10%, 5%), embedded at 60 °C in paraffin and cut in 4 μm thick sections with a Rotary Microtome HM 355 S (MICROM International GmbH, Walldorf, Germany).

The sections were floated onto distilled water and heat-stretched in 50 °C distilled water. The sections were mounted onto positively charged slides; superfrost® Plus slides (Menzel-Gläser, Braunschweig, Germany). The sections were heated in an oven (30 min, 60 °C) and allowed to cool to RT. Paraffin was removed in xylene, followed by rehydration in a graded series of ethanol (99.5%, 95%, 70%) and washed in 0.1 M PBS. The slides were allowed to reach boiling temperature in 10 mM citrate buffer (pH 6.0) and were boiled for 15 min in a microwave oven at 750 W.

The slides were allowed to cool before washed in PBS (3 × 3 min). The tissue was blocked with 3% goat serum (Sigma–Aldrich) and

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2.5% non-fat dry milk in 0.1 M PBS (30 min, RT). The tissue was washed in PBS (3 × 3 min) and inhibition of endogenous peroxidases was performed by the addition of Dako® Peroxidase Blocking Reagent (Dako, Carpinteria, CA; 10 min) or 0.3% hydrogen peroxide in 0.1 M PBS (30 min) onto the tissues. The slides were washed in PBS (3 × 3 min) and were incubated with the anti-PDX antibody (1 h, RT, or over night at 4 °C in a humidified chamber). The slides were washed in PBS (3 × 3 min) and incubated with the secondary biotinylated antibody; Rabbit IgG (Vectastain ABC-kit, Vector laboratories, CA; 60 min, RT). The slides were washed in PBS (3 × 3 min). The primary and secondary antibodies were diluted in the previously used blocking solution. A vectastain Elite ABC-kit (Vector Laboratories, Inc., Burlingame, CA) was applied to the tissue in order to detect the secondary antibody (30 min, RT). The slides were washed with PBS (3 × 3 min). A DAB/peroxidase detection kit was used for development (Vector laboratories), and applied until a black reaction product was seen. The slides were washed once more in PBS (3 × 3 min) and were then dehydrated in a graded series of ethanol (70%, 95%, 99.5%) and subsequently immersed in xylene. The slides were finally mounted and evaluated in a light microscope.

2.11. Determination of pyridoxine content

Pyridoxine was extracted from *Arabidopsis* leaves exposed to UV-B for 3 days in addition to control samples using a modified protocol from Kall [28] and van der Berg et al. [29]. 0.5 g of leaf material was ground with a mortar and pestle in 10 ml of 0.22 M H_2SO_4 and autoclaved at 121 °C. The debris was removed by centrifugation for 10 min at 5000 rpm and the pH was brought to 4.8 by using NaAc. The extract was filter-sterilized using a 0.22 μM filter (Millipore, MA, USA) and used directly for analysis. The amount of pyridoxine was determined by using the microbiological assay as described by Gregory [30], using *Saccharomyces cerevisiae* (ATCC#9080). The assay was set up using 10 μl of leaf extract and each sample was run in triplicate.

3. Results

3.1. Production of recombinant PDX1.3 protein and antibodies against the protein

As investigated using DNA array analysis, the *PDX1.3* mRNA was one of the strongest and most specifically induced transcripts after exposure of wild type *A. thaliana* to UV-B radiation (Col-0 ecotype; Ref. [10]). It was thus of interest to investigate whether the protein itself was similarly induced in wild type tissue. Therefore, recombinant PDX1.3 was obtained and used for antibody production. When producing recombinant PDX1.3 in *E. coli*, the protein was found in the soluble fraction, and typically 2 mg recombinant PDX1.3 protein l^{-1} growth medium was obtained. When analysing the purified protein with SDS–PAGE (Fig. 1a), its size was estimated to approximately 33 kDa (theoretical molecular weight with a His-tag is 34 038 Da), and only weak bands of contaminating proteins were visible. A sample of the purified protein was sent to the Karolinska Institute Protein Analysis Centre (Solna, Sweden) for C-terminal sequencing and amino acid analysis. The two C-terminal serine and glutamic acid residues were confirmed by sequencing, and the amino acid composition of the purified protein closely corresponded to that expected for the PDX1.3 protein (not shown). To further confirm the identity of the purified protein we used an enzyme activity assay, where the pyridoxal 5-phosphate (PLP) synthase activity was monitored. The purified protein was active (Fig. 1b), i.e. an increase in absorbance at 414 nm (due to the accumulation of synthesized PLP) was observed. The estimated activity of the purified protein was 1.7 nmol PLP min^{-1} mg^{-1} protein. There was no increase in absorbance at 414 nm when the recombinant protein was absent from the solution, regardless of the order of addition of the different substrates. The activity was in agreement with what was previously reported by Tambasco-Studart et al. [17]. After confirming the identity of our recombinant PDX1.3 protein, both native and SDS-treated protein samples were sent for antibody production.

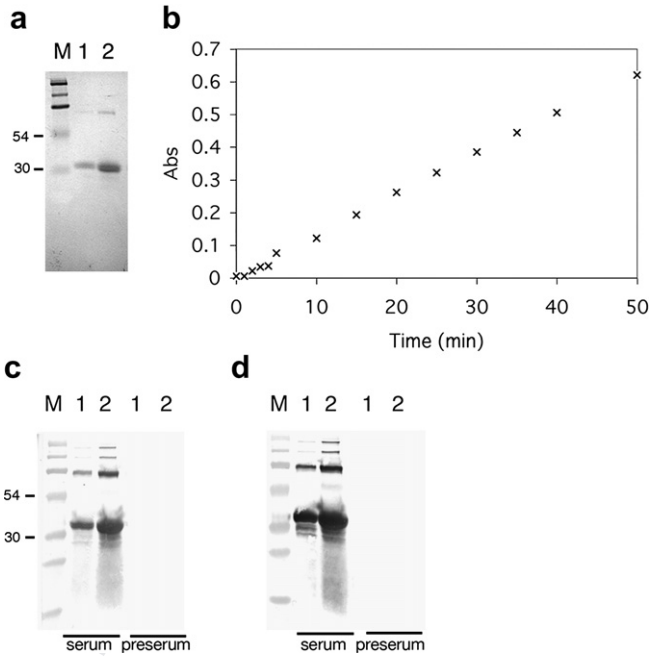

Fig. 1. (a) SDS–PAGE showing purified recombinant PDX1.3 protein. (b) Pyridoxal 5-phosphate synthase activity assay of purified recombinant PDX1.3. The reaction mixture contained 500 μM ribose 5-phosphate, 1 mM glyceraldehyde 5-phosphate, 10 mM ammonium sulphate and 1.33 mg ml^{-1} of isolated recombinant PDX1.3 protein. The build up of pyridoxal 5-phosphate was recorded as the increase in absorbance at 414 nm at discrete time-points. The reaction was carried out at 37 °C. (c) Western blot using either rabbit antiserum raised against native recombinant PDX1.3 or preimmune serum. (d) Western blot using either rabbit antiserum raised against SDS-treated recombinant PDX1.3 or preimmune serum. For (a, c and d): Molecular weight marker (lane M), 0.11 μg of recombinant PDX1.3 loaded (lane 1), 0.32 μg of recombinant PDX1.3 loaded (lane 2).

To test the binding capability of the resulting PDX1 antibodies, two different amounts (0.11 and 0.32 μg) of purified recombinant PDX1.3 protein were separated and transferred onto nitrocellulose membranes. As is seen, antibodies raised against both the native (Fig. 1c) and the SDS-treated proteins (Fig. 1d) recognized the recombinant protein. However, antibodies raised against SDS-treated PDX1.3 gave a somewhat stronger signal than the antibodies raised against native PDX1.3 and therefore used in the remainder of the study. None of the presera reacted with any proteins (Fig. 1c and d; preserum lanes 1 and 2).

3.2. Expression and tissue localisation of PDX1

Purified antibodies were used to study the induction of the PDX1 protein after UV-B exposure, and, in addition, to roughly determine the protein localisation to either the cytosolic fraction or the membrane subcellular fraction. The latter was of importance due to a controversy in the literature as to the subcellular localisation of the protein [12,17,31]. The protein levels were studied using Western blotting (Fig. 2a and b). PDX1 protein could be detected in both the cytosolic and the membrane subcellular fractions. In the cytosolic fraction (Fig. 2a), the protein was induced 1 day after commencement

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of the exposure (6 h UV-B radiation every 24 h) in the Col-0 ecotype compared with UV-A-treated Col-0. The protein level remained at a higher level in the extract from leaves of UV-B-treated plants during the whole period of exposure, i.e. for 7 days (Fig. 2a). The PDX1 protein was present at low levels in the membrane fraction of *Arabidopsis* leaves (Fig. 2b). Similar to what was found in the cytosolic fraction, higher amounts of protein were observed in the UV-B-exposed plants. Infrequently, some PDX1 proteins were also found in the cytosolic fraction of untreated plants (exemplified by the occurrence of a band in the 0 h time-point in Fig. 2a).

In addition, to confirm that the protein labelled by the antibodies in *Arabidopsis* leaves exposed to UV-B indeed was a PDX1 protein, immunoprecipitation was performed followed by analysis with SDS–PAGE. A clear band was obtained (Fig. 2c) that was further excised and identified by in-gel trypsin treatment and MS analysis to correspond to the PDX1.1 protein (Fig. 2d). In addition to this identification, these results also show that the antibodies have a high affinity for both PDX1.1 (Fig. 2c) and PDX1.3 (against which the antibodies were raised; Fig. 1c and d), which would be expected considering the amino acid identity score of 87% between the two proteins.

To further evaluate the localisation of PDX1 protein in plant tissue, immunohistochemical analysis was employed using the PDX1-specific antibodies. For this experiment, *P. sativum* plants were used since we were not able to obtain high quality slices of *Arabidopsis* tissue suitable for immunohistochemistry. For confirmation of the usefulness of the PDX1 antibodies also for labeling pea PDX protein, the experiment shown in Fig. 3a was set up. A good cross-reaction was obtained on western blots with a protein of a size similar to the recombinant *Arabidopsis* protein. Also similar to the case in *Arabidopsis*, this protein was expressed after 3 days of UV-B exposure.

Using the PDX1 antibodies, immunohistochemical staining of slices of leaves from pea plants exposed to UV-B for 5 days (Fig. 3b). PDX1 was found concentrated to the palisade parenchyma cells, whereas the upper epidermis did not show any staining. Also, the protein was excluded from the vacuoles. Sliced pea leaves incubated with preimmune serum did not show any staining (Fig. 3c).

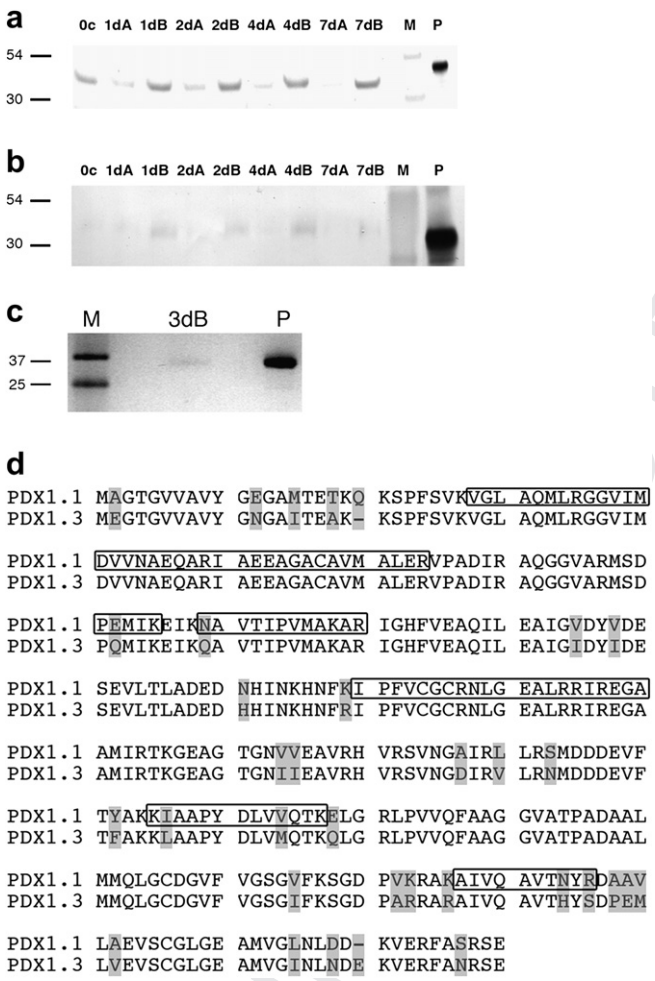

Fig. 2. Western blot showing the presence of the PDX1 proteins in the leaves of *Arabidopsis thaliana* Col-0 wild type. Leaf extracts were from controls (0 h) or plants exposed to either UV-A (0.31 W m^{-2}) or UV-B (UV-$B_{BE,300}$ = 0.18 W m^{-2}) for 6 h every 24 h. For UV-A (A) and UV-B (B) irradiation, the exposures were carried out for 1 day (1dA, 1dB), 2 days (2dA, 2dB), 4 days (4dA, 4dB) or 7 days (7dA, 7dB). In addition, a molecular weight marker standard (M) and the recombinant PDX1 protein (positive control; P) were loaded. (a) Total protein from the leaf cytosolic fraction. 12 μg was loaded in each lane. (b) Proteins from the leaf membrane fraction. Samples were loaded at a concentration of 5.5 μg of Chl in each lane. (c) Immunoprecipitation of PDX1.1 protein in the leaves of the *Arabidopsis thaliana* Col-0 wild type exposed to UV-B (UV-$B_{BE,300}$ = 0.18 W m^{-2}) for 6 h for 3 days (3dB). (d) Amino acid sequence alignment of PDX1.1 and PDX1.3 from *Arabidopsis*. Differences between the sequences are shown by shading. Boxed are the sequences obtained by MALDI-TOF mass spectrometry analysis of the band excised from c (3dB), exactly matching peptides from the PDX1.1 sequence.

3.3. Pyridoxine content after UV-B exposure

To ascertain whether the higher PDX1 protein levels after UV-B exposure were reflected in higher levels of pyridoxine, the content of this compound was measured in leaves of Col-0 wild type plants after 3 days of UV-B treatment (Fig. 4a). The irradiation led to a strong increase (by 62%) of the concentration of pyridoxine in Col-0 compared with the unexposed or UV-A-exposed plants. In an *Arabidopsis pdx1.3* knock-out mutant, the same UV-B exposure did not lead to any increase in the pyridoxine levels (Fig. 4b).

3.4. Molecular marker analysis in the Arabidopsis pdx1.3 mutant and in the Col-0 wild type

To further investigate the involvement of pyridoxine in UV-B radiation responses, knock-out mutants of the *pdx1.3* gene were

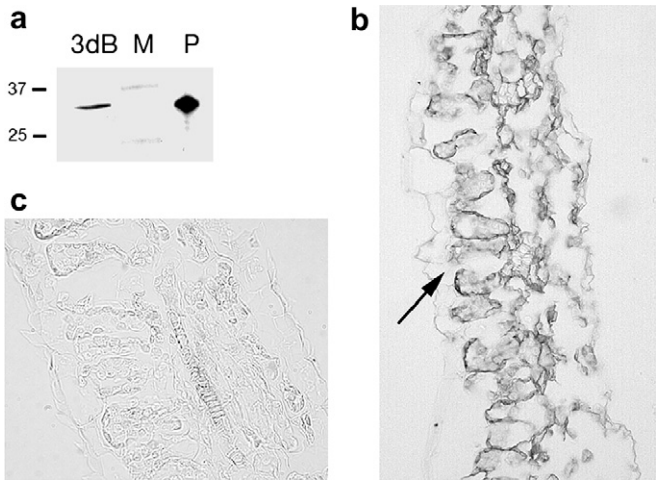

Fig. 3. (a) Western blot showing the presence of the PDX1 protein in pea leaves exposed to 3 days of UV-B (UV-$B_{BE,300}$ = 0.18 W m^{-2}) for 6 h each 24 h (3dB). In addition, a molecular weight marker standard (M) and the recombinant *Arabidopsis thaliana* PDX1.3 protein (positive control; P) were loaded. (b–c) Immunohistochemical staining of sections of leaves from *Pisum sativum*. Plants were exposed to UV-B (b; UV-$B_{BE,300}$ = 0.3 W m^{-2}) radiation for 5 days (6 h every 24 h). Tissue sections were analysed using either primary polyclonal SDS–PDX1.3 antiserum (diluted 1:2000; b) or preimmune serum (c). The arrow indicates the cells of the upper epidermis facing the radiation.

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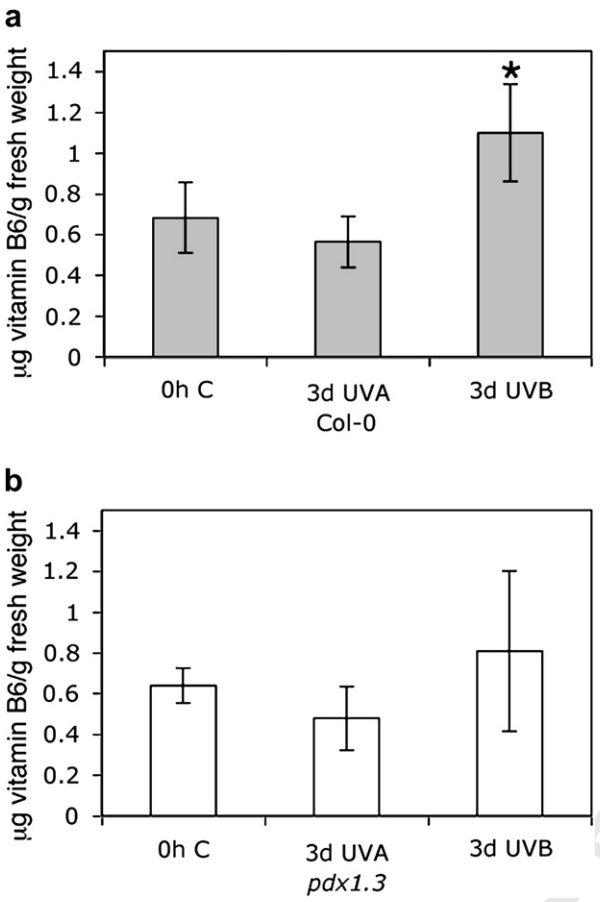

Fig. 4. Vitamin B_6 measurements in *Arabidopsis* Col-0 wild type (a) or *pdx1.3-1* mutant (b) plants exposed to 3 days of either UV-A (0.31 W m^{-2}) or UV-B (UV-$B_{BE,300}$ = 0.18 W m^{-2}) for 6 h every 24 h. Means from three independent experiments, each performed in triplicate, are shown. Error bars show the SD of the mean. Bar labelled * is significantly different from the control sample (p-value < 0.005).

studied for their capability of expressing four molecular UV-B markers. For the mutants *pdx1.3-1* and *pdx1.3-2*, similar transcription patterns were obtained and therefore only *pdx1.3-1* is presented (Fig. 5). The UV-B markers *PR-5*, *CHS*, *MEB5.2* and *LHCB1*3*, were chosen to reflect different signalling pathways for the UV-B response [32]. Whereas *PR-5* (encoding the pathogenesis-related protein 5) represents the oxidative stress pathway [7], *CHS* (encoding chalcone synthase, an important enzyme participating in the formation of UV-B-absorbing flavonoids) belongs to the UV-B-specific pathway [33]. *MEB5.2* was regulated by the lowest UV-B levels of any gene in the study by Brosché et al. [10] and *LHCB1*3* is encoding a chlorophyll *a*/*b*-binding protein of the photosystem II light-harvesting antenna complex and is a representative of genes known to be down-regulated by UV-B [8].

The oxidative stress marker *PR-5* was expressed differently between the mutants and the wild type plant after exposure to UV-B radiation (Fig. 5a). It was induced after 3 h of exposure (3 h UV-B) both in the mutant and in Col-0, although the expression level was higher in the *pdx1* mutants (25% higher transcription level in the *pdx1.3-1* mutant compared with Col-0). After 9 h of UV-B irradiation, the mRNA levels in the Col-0 plants increased to a similar level as in the mutant. UV-A radiation also induced *PR-5*, and the mutant showed a higher level of expression than control plants after 9 h of UV-A radiation (60% higher than Col-0).

In contrast, both the UV-B-specific marker *CHS* (Fig. 5b) and the low level UV-B marker *MEB5.2* (Fig. 5c) showed no differences in their expression patterns when we compared the mutants with the

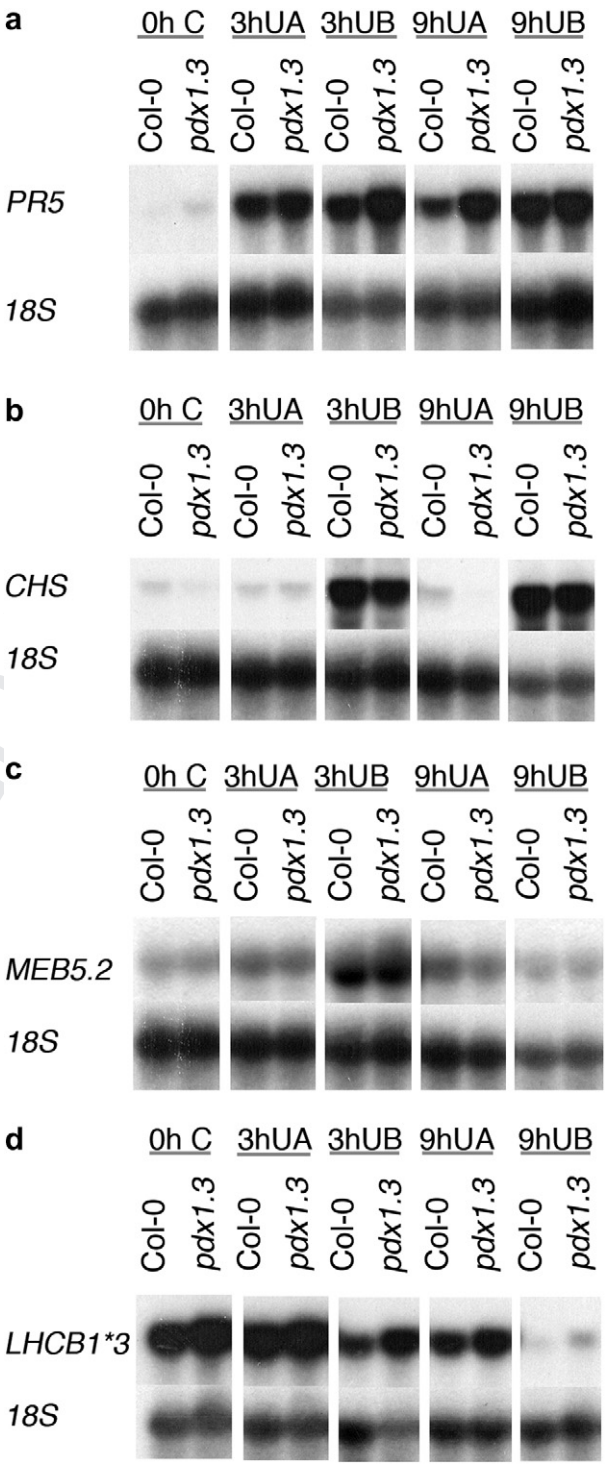

Fig. 5. Autoradiographs of Northern blots hybridized with [^{32}P]-labelled cDNA probes for (a) *PR-5*, (b) *CHS*, (c) *MEB5.2*, (d) *LHCB1*3*, and 18S rRNA. 10 µg of total RNA isolated from Col-0 wild type and *PDX1* knock-out mutant (*pdx1.3-1*) was loaded in each lane. The plants were exposed to UV-A (0.31 W m^{-2}) or UV-A + UV-B (UV-$B_{BE,300}$ = 0.18 W m^{-2}) for 0 h (0C), 3 h (UV-A, UV-B) or 9 h (UV-A, UV-B). To control even loading and transfer of total RNA, each membrane was reprobed with the 18S rRNA probe.

wild type. The transcripts were present at very low levels in the control plants and after UV-A irradiation (0 h C, 3 h UV-A, 9 h UV-A). However, after UV-B irradiation for 3 and 9 h respectively, the CHS transcript was induced to a similar high level in both mutants and in the Col-0 wild type (Figs. 5b; 3 h UV-B, 9 h UV-B).

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For *MEB5.2*, a transient expression was seen after UV-B exposure. The mRNA levels increased after 3 h of exposure and thereafter returned to basal levels at 9 h (Fig. 5c; 3 h UV-B, 9 h UV-B).

The *LHCB1*3* transcript was present at almost similar levels in non-exposed control plants and in the corresponding mutants (Fig. 5d; lane 0 h C). After 3 h of exposure to ultraviolet-B radiation (3 h UV-B), the mRNA was strongly down-regulated in the wild type plants, 60% transcript reduction compared with UV-A control. However, in the mutants, the expression levels of *LHCB1*3* were not reduced to the same extent, only a reduction of 11% was measured for the *pdx1.3-3* mutant compared with the wild type. The same trend was also seen after 9 h of UV-B radiation, where the *LHCB1*3* transcripts had almost vanished in the Col-0 plants, but were still present in low amounts in the mutants (9 h UV-B, 2–3 times more in the mutants than in Col-0). Also, in the UV-A controls, the mutants had higher *LHCB1*3* mRNA levels than Col-0.

4. Discussion

The involvement of reactive oxygen species (ROS) in plants after UV-B irradiation has previously been inferred. Increased activities of anti-oxidative enzymes have been demonstrated, in addition to decreased content of antioxidants [6]. Also, changes in expression of ROS-dependent genes, such as *PR-1* and *PR-5*, indicate that ROS is involved in at least high level or long-term UV-B responses [7,32,34]. It was therefore of importance that Brosché et al. [10] observed the up-regulation of *PDX1.3*, indicating an important role for the gene product under UV-B irradiation. The gene is known to be needed for resistance in *Cercospora nicotianae* towards the singlet oxygen photosensitizer cercosporin [13] (Ehrenshaft et al., 1999), and later studies have shown that the product of the PDX1 enzymatic reaction, pyridoxine (vitamin B_6), also has a broader quenching capacity for different ROS species [12,35,36].

In order to evaluate the role of PDX1 in the UV-B response, its protein levels and the vitamin B_6 content were determined in *A. thaliana* Col-0 wild type and *pdx1.3* mutants before and after exposure. In addition, mutants were also used to study the involvement of the *PDX1* gene in UV-B-dependent regulation of expression of a number of key molecular markers.

By using PDX1-specific antibodies, we were able to show an increased level of the PDX1 protein in plants exposed to UV-B radiation compared with plants exposed to UV-A alone (Fig. 2a and b). That the labelled protein was indeed PDX1 was shown by immunoprecipitation and mass spectrometry analysis (Fig. 2c and d). This is in agreement with the reports by Brosché et al. [10], Ulm et al. [11], Brown and Jenkins [37], and Favory et al. [38], where the *PDX1.3* transcript levels were shown to increase after UV-B treatment. In the three latter studies, these data are presented in their corresponding Supplementary material.

There have been contradictory reports as to where PDX1 is located in the cell, whether it is a cytosolic protein or is membrane-associated. Using GFP-PDX1 fusion protein constructs, Chen and Xiong [12] concluded that the protein is mainly membrane-associated, although they did not rule out that some proteins might be cytosolic. In a separate study, also using GFP–PDX1 fusion protein constructs, Tambasco-Studart et al. [17] concluded that PDX1 is cytosolic. In the present study, when separating the cytosolic proteins from membrane-associated proteins, we found that the PDX1 antibodies cross-reacted with proteins isolated from both the cytosolic fraction (Fig. 2a) and the membrane fraction (Fig. 2b) although the majority of the protein was present in the cytosol. As concluded by Chen and Xiong [12], the membrane-associated PDX1 might have a role in protection against membrane lipid peroxidation. High doses of UV-B radiation are known to lead to membrane damage [3] most likely as a result of lipid peroxidation. The cytosolic PDX1 protein in turn may be involved in more general pyridoxine-induced quenching of intracellular ROS.

It should also be noted, that sometimes PDX was present in the 0 h non-irradiated control to a level that was higher than for UV-A-irradiated samples (Fig. 2a). This observation was not always found and the reason is not known. Notwithstanding, PDX1 levels were always highest in UV-B-irradiated leaves.

To further examine the tissue distribution of PDX1 in plants, we used the non-quantitative method of immunohistochemistry to localise the protein in *P. sativum* leaves. In pea, PDX1 was found towards the UV-B-exposed side of the leaf. A need for more PDX protein close to the irradiation site in case the radiation should give rise to ROS could be the cause for this shift. Alternatively, there could be a greater need for PLP (the product of the enzymatic activity of the PDX1/2 complex and a common co-factor for e.g. amino acid metabolizing enzymes) close to irradiated tissue, as a result of UV-B-dependent alteration of metabolism.

To study the role of PDX1 in the UV-B-dependent regulation of gene expression, four molecular markers were used in this study, representing different pathways of the UV-B-induced signalling pathways [32]. *PR-5* is known to be induced both by high levels of UV-B radiation and by ROS and is also used as an oxidative stress marker. The *CHS* and *MEB5.2* molecular markers are more specifically induced by low level UV-B [7,33] and are classified as genes induced by low and very low doses of UV-B, respectively [32]. Genes corresponding to chloroplast-localized proteins are known to be down-regulated by UV-B [9] and are here represented by *LHCB1*3*.

The different markers showed disparate regulation patterns with respect to the functionality of the *PDX1* gene. For *PR-5*, a higher transcription level was found in *pdx1.3* primarily after 3 h of UV-B radiation (Fig. 5a), and, apparently, the non-specific UV-B-signalling pathway, represented by this gene [32], was active to a higher level in the *pdx1.3* mutants. *PR-5* transcripts were also present in non-irradiated plants to some extent and, consequently, the *pdx1.3* mutants might be constantly de-repressed with respect to this signalling pathway, possibly a result of low levels of oxidative stress also under normal growth conditions.

There was no difference in regulation of the *CHS* and *MEB5.2* genes when we compared the mutant and the wild type plants (Fig. 5b and c). Apparently, these genes, representing low and very low level UV-B-specific signalling pathways, respectively, were not affected by the non-functional *PDX1* gene. This further confirms the induction of these genes as a UV-B-specific event, and not a result of formation of reactive oxygen species.

Down-regulation of *LHCB1*3* expression could be seen in both the *pdx1.3* mutants and in Col-0 wild type (Fig. 5d). Still, the RNA blot showed higher levels of *LHCB1*3* transcripts in the *pdx1.3* mutants compared with the Col-0 wild type. This was found both in plants grown under control conditions and in plants exposed to UV-B, but it was most apparent after 3 h of UV-B.

Although the *pdx1.3* mutants showed no *PDX1.3* transcripts on a Northern blot (not shown), we were able to detect a protein of approximately the correct size using our antibodies towards PDX1.3 in the mutant (not shown). The two *Arabidopsis* proteins, PDX1.1 and PDX1.3, are too similar to be discriminated using our antibodies and we probably detect the functional PDX1.1 protein in the mutants. Recently, Wagner et al. [20] concluded that both genes probably have overlapping functions, and both are required to act synergistically to establish pyridoxine homeostasis. Also, comparable basal levels of vitamin B_6 were detected in both the *pdx1.3* mutant and in the Col-0 ecotype. After exposure to UV-B, significantly increased vitamin B_6 levels were found in Col-0 wild type (Fig. 4a) but not in the mutant (Fig. 4b). Most likely, under all conditions in the *pdx1.3* mutant and in Col-0 under control

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conditions and under UV-A exposure, the basal pyridoxine level is provided by the ubiquitous PDX1.1 homologue, whereas PDX1.3 is responsible for the additional pyridoxine found in Col-0 under UV-B exposure. Strengthening this is the fact that studies showing UV-B-induced expression of the PDX1.3 gene failed to show any regulation of the PDX1.1 gene whatsoever [10,11,37,38].

We have summarized in the model in Fig. 6 the knowledge regarding UV-B regulation of the PDX1 and pyridoxine levels and their influence on other UV-regulated genes. Transcription of the *PDX1.3* gene is accomplished by low level UV-B radiation in the waveband between 300 and 310 nm [39] by a putative UV-B receptor. This receptor would regulate PDX1.3 expression via the UVR8 and COP1 components since corresponding mutants lack UV-B induction of PDX1.3 (Ref. [38] and supplementary material connected to that publication). PDX1.3 transcription is to a large extent impaired in mutants deficient in brassinosteroid synthesis [25] and in NADPH oxidase mutants [40]. These enzyme systems are therefore needed for full *PDX1.3* gene expression. In this paper we show that the PDX1 protein levels are increased by UV-B and so is accumulation of pyridoxine, the product of the PDX1/PDX2 heterododecamer enzyme. PDX1 or pyridoxine in turn is needed for the wild type regulation of *PR-5*, a gene non-specifically regulated by UV-B and other stimuli [23], i.e. the *pdx1.3* mutant is derepressed in PR-5 expression. Also, the UV-B-dependent down-regulation of the photosynthetic *LHCB1*3* gene is considerably smaller in *pdx1.3*, partially blocking signalling from the putative UV-B receptor absorbing radiation in the 280–290 nm waveband which controls expression of another subset of UV-B-regulated genes [39], including *LHCB1*3*.

In conclusion, and based on our results, we conclude that PDX1 is involved in the response of plants towards UV-B irradiation since it is induced both at the mRNA and the protein levels, and since pyridoxine levels rise upon UV-B exposure. Furthermore, UV-B-induced PDX1 formation occurs mainly in the part of the leaf where UV-B radiation is absorbed. Also, the medium and high UV-B level signalling pathways are altered in the *pdx1.3* mutant as suggested by alteration in *LHCB1*3* and *PR-5* gene expression.

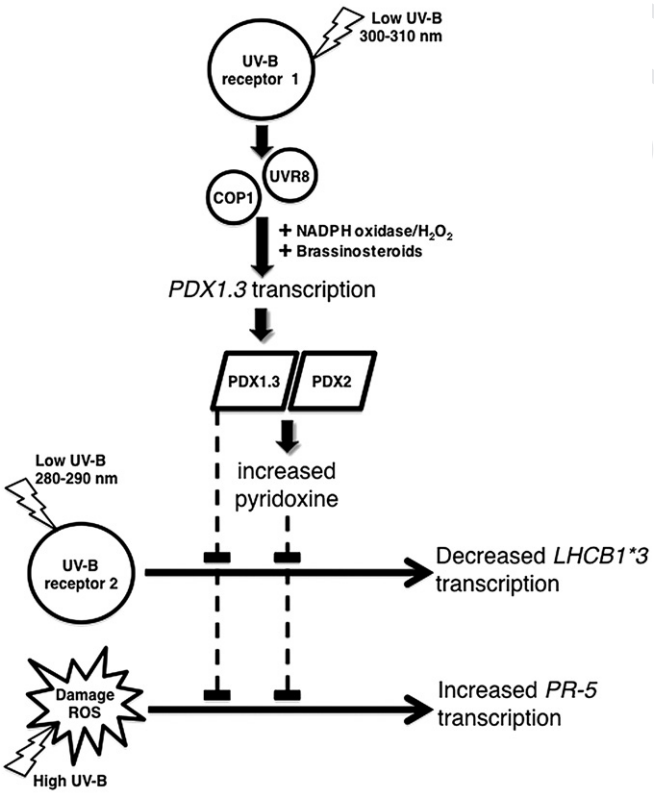

Fig. 6. A model showing regulation of *PDX1* gene expression and pyridoxine production by UV-B. Absorption of UV-B quanta between 300 and 310 nm leads to a signal that is transduced by the UVR8 protein to induce transcription of the *PDX1.3* gene. For UV-B-induced *PDX1.3* expression, the COP1 component is also obligatory. Brassinosteroids and NADPH oxidase/H_2O_2 are necessary for full *PDX1.3* gene expression. In plants containing a functional *PDX1.3* gene, other UV-B-signalling pathways, inducing *PR-5* gene expression and down-regulation of the *LHCB1*3* gene, respectively, are repressed. This repression, which can be dependent either on pyridoxine or on the PDX1.3 protein itself, is lifted in the *pdx1.3* mutant.

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