

Regulation of biosynthetic genes and antioxidant properties of vitamin B₆ vitamers during plant defense responses

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Abstract

Vitamin B₆, an essential cofactor in enzymatic reactions, has only recently been linked to cellular oxidative stress. We investigated the role of this vitamin as an antioxidant in oxidative responses linked to plant defense. B₆ vitamers effectively quenched superoxide and had antioxidant activity when assayed in vitro. The de novo B₆ biosynthetic genes (*PDX1* and *PDX2*) were identified in *Nicotiana tabacum* cv. 'Burley 21' and their transcript abundance was assayed during defense responses. *PDX1* and *PDX2* transcript levels decreased following inoculation with the incompatible pathogen *Pseudomonas syringae* pv. *phaseolicola* and transiently increased in response to salicylic acid and methyl jasmonate. Excess vitamin B₆ in tobacco leaves interfered with the development of a hypersensitive response caused by *P. syringae* pv. *phaseolicola* and increased disease severity caused by the compatible bacterium *P. syringae* pv. *tabaci*. Our findings indicate that during plant defense responses, vitamin B₆ functions and its synthesis is regulated in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species in vivo.

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1. Introduction

Vitamin B₆, the collective name given to pyridoxine, pyridoxamine, pyridoxal and their phosphorylated derivatives, is an essential cofactor for numerous enzymatic reactions. It is most notable for its contribution to amino acid biosynthesis where it serves as a cofactor for enzymes involved in decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions [24,48]. Other significant functions include its involvement in carbohydrate and lipid metabolism, in producing some antibiotic precursors, and in synthesizing aminocyclopropane-1-carboxylate (ACC) [24,48].

The vitamin B₆ pathway is poorly characterized in plants. Vitamin B₆ biosynthesis has been thoroughly characterized in *Escherichia coli* and involves a de novo pathway that produces pyridoxine 5'-phosphate as well as a salvage pathway that interconverts between the different vitamers [4,18,24,32–34,

41–43,46,52,54,63]. Our lab and others have recently documented that plants, fungi, archaeobacteria, and most eubacteria use a distinct de novo biosynthetic pathway involving two genes, *PDX1* and *PDX2*, that have no homology to the *E. coli* de novo biosynthetic genes *pdxA* and *pdxJ* [25,26,48]. To date, little is known about the enzymes encoded by these genes or the substrates in this pathway. The *PDX2* gene product has recently been shown to be a glutaminase [6,21], and is hypothesized to be involved in production of the nitrogen-containing substrate for the *PDX1* protein. It is known that *PDX1* and *PDX2* proteins form a complex [21], but the *PDX1* sequence provides no clues to its function. We recently demonstrated that *PDX1* complements *pdxJ*, and to a more limited extent *pdxA*, mutations in *E. coli*, strongly suggesting that *PDX1* catalyzes the ring closure reaction of the pyridoxine molecule [60].

The importance of vitamin B₆ as a cofactor is well established, but it is only in the last decade that research has linked vitamin B₆, often unknowingly, to oxidative stress. The earliest suggestions of a connection between vitamin B₆ and cellular antioxidant defense were based on regulation studies of homologues of *PDX1* and *PDX2* [25,26]. In yeast (*Saccharomyces cerevisiae*), the *PDX1* and *PDX2* homologues, *SNZ1*

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and *SNO1*, show increased transcript and protein accumulation at entry into stationary phase, a time of high oxidative stress [11,50]. In the bacterium *Bacillus subtilis*, there is increased protein accumulation of the *PDX1* homologue upon treatment with paraquat, an inducer of superoxide [3]. H_2O_2 treatment of *Schizosaccharomyces pombe* leads to increased transcript accumulation of the *PDX2* homologue as well as pyridoxal reductase, a gene whose product is part of the B_6 salvage pathway [13]. Pyridoxal kinase, another component of the salvage pathway, has been connected to salt tolerance and cold response in *Arabidopsis thaliana* [55], and one of the *Arabidopsis PDX1* homologues (on chromosome 5) shows increased transcript accumulation after exposure to UV-B radiation [12].

In addition to gene regulation studies, metabolic evidence has also been mounting that vitamin B_6 is an essential antioxidant and a strong quencher of active oxygen species. In previous work, we showed that vitamin B_6 is a potent quencher of singlet oxygen, with quenching rates comparable to or greater than those of vitamins C and E, two of the most efficient biological antioxidants known [7]. Vitamin B_6 biosynthetic genes are necessary for resistance to singlet oxygen-generating photosensitizers in the fungus *Cercospora nicotianae* [27]. Also, a recent study in *Arabidopsis* showed that vitamin B_6 decreases singlet oxygen-induced death in *flu* mutant protoplasts [14]. The antioxidant activity of vitamin B_6 is corroborated by findings from animal models. In blood assays, vitamin B_6 had three times the antioxidant activity of vitamin C [57] and was shown to quench superoxide production [35,37]. Vitamin B_6 prevented protein oxidation in rabbit lens cells (a cause of cataract formation) [36], and reversed the elevated inflammatory response and lipid peroxidation characteristic of vitamin B_6 -deficient rats [40]. Clinical trials have shown that vitamin B_6 supplements prevent or delay eye and nerve damage associated with diabetes and attributed to superoxide production [35].

In 1995, a report was published showing that treatment of rubber tree (*Hevea brasiliensis*) with salicylic acid and ethephon, chemical inducers of plant-pathogen defense responses, boosted transcript accumulation of what are now known to be *Hevea* homologues of *PDX1* [56], suggesting that vitamin B_6 may be important during plant defense responses. During pathogen attack, plants produce active oxygen species (AOS) such as superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide. AOS play a central role in plant defenses to pathogen attack, acting as antimicrobial agents, as substrates in cell wall fortification, and as signaling molecules for the activation of defense pathways [5,8,15,20,29,44,45,47]. AOS production must be tightly controlled as over-accumulation of AOS can result in unwanted cell death. Thus, plants must sustain AOS levels at the site of infection to ward off pathogens and to stimulate the defense response, yet keep AOS levels lower in regions not affected by the pathogen to maintain tissue integrity and viability. To help with this fine-tuning, plants employ AOS scavenging enzymes, such as ascorbate peroxidase and catalase, and metabolites including glutathione, ascorbic acid, and α -tocopherol, during pathogen defense [49].

Since the study on *Hevea PDX1* gene expression, no further studies on vitamin B_6 and plant defense responses have been published. We were interested in defining the possible role of vitamin B_6 in plant defense responses. In addition, the requirement for AOS for successful plant defense suggested to us that the hypersensitive response (HR) may provide an ideal system to confirm the in vivo activity of vitamin B_6 as an antioxidant. Here, we report on the ability of B_6 vitamers to quench superoxide and prevent lipid peroxidation, the regulation of B_6 -synthesizing genes following salicylic acid and methyl jasmonate treatment and pathogen inoculation, and the effect of elevated levels of pyridoxine on the course of defense responses in tobacco. Our results show that vitamin B_6 can act as an antioxidant in planta, and that vitamin B_6 biosynthetic genes are regulated during plant-pathogen defense responses in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species in vivo.

2. Materials and methods

2.1. Superoxide quenching assay

B_6 vitamers, pyridoxine, pyridoxal, and pyridoxamine, were tested for their ability to quench superoxide generated via a xanthine–xanthine oxidase reaction and monitored through the superoxide-mediated reduction of cytochrome *c*. The reaction mixture contained a final concentration of 50 mM KH_2PO_4 , 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 mM cytochrome *c* (Sigma, St Louis, MO), and 0.05 mM xanthine (Sigma) at pH 7.8. Pyridoxine, pyridoxal and pyridoxamine (Sigma) were added to this solution at final concentrations of 0.1, 1, 10 and 50 mM, and solutions were re-adjusted to a pH of 7.8. Xanthine oxidase (0.0025 units) (Sigma) was added immediately before measurement. All reactions were carried out at 25 °C in a total volume of 1.5 mL. The reaction was measured at 15 s intervals for 5 min using a Beckman DU 650 spectrophotometer to measure A_{550} against a blank lacking xanthine oxidase. Percent inhibition was calculated by the following equation based on a linear slope:

$$\% \text{ inhibition} = \frac{\text{slope uninhibited} - \text{slope inhibited}}{\text{slope uninhibited} - \text{slope blank}} \times 100$$

Superoxide dismutase (SOD) (Sigma) was used as a control for the assay. In our assay, 0.25 μ g SOD (defined by the manufacturer Sigma as equal to one unit) in a final reaction volume of 1.5 ml, resulted in approximately 50% inhibition. Each assay was repeated three times. Statistical differences were determined using a *t*-test.

2.2. Lipid peroxide quenching assay

The antioxidant activity of B_6 vitamers, pyridoxine, pyridoxal, and pyridoxamine, was tested in a spectrophotometric assay measuring inhibition of the coupled oxidation of β -carotene and linoleic acid as described by Hammerschmidt and Pratt [31] and Daub [16]. The reaction mixture contained

0.1 mg β -carotene (Sigma), 20 mg linoleic acid (Sigma) and 200 mg Tween 40 in 1 mL of chloroform. The chloroform was removed using a rotary evaporator and 50 mL of oxygenated distilled water was added to the residue. Five milliliters of this mixture was combined with 0.2 mL of vitamin B₆ stock solutions (final concentrations of 4, 40, 400 μ M and 4 mM) and incubated at 50 °C. The amount of antioxidant activity was determined by measuring the change of absorbance at 470 nm at 15 min intervals for 90 min against a blank lacking β -carotene. Percent inhibition was determined based on the linear slope using the equation above in Section 2.1. Statistical differences were determined using a *t*-test.

2.3. Plant materials and growth conditions

Nicotiana tabacum cv. ‘Burley 21’ was grown under green house conditions in clay pots or plastic packs in a 4:1 mixture of Metro Mix (Scotts-Sierra Horticultural Products Co., Marysville, OH) and soil (Rex Frazier, Sanford, NC) with Osmocote fertilizer (Scotts-Sierra Horticultural Products Co.) for approximately 8 weeks. One week before experiments, plants were brought to the lab and placed on a plant rack with 100 μ mol s⁻¹ m⁻² light with a 16 h photoperiod for pathogen tests or in an incubator with 100 μ mol s⁻¹ m⁻² light, 25 °C with a 16 h photoperiod for chemical treatments.

2.4. Chemical treatment

For salicylic acid treatment, upper and lower surfaces of leaves of 8–10 week old plants were lightly sprayed with 2 or 5 mM salicylic acid (Sigma) or with deionized H₂O. At each time point (0, 6, 24, 48 h) all leaves (cut 1 in. above the petiole) were removed from three separate plants, bulked, and immediately frozen in liquid nitrogen and stored at –80 °C. The salicylic acid treatment experiment was repeated once. For methyl jasmonate treatment, upper and lower surfaces of leaves were lightly sprayed with 1 mM methyl jasmonate (Sigma). Leaves were harvested and stored as described above.

2.5. Pathogen treatment

The terminal halves of tobacco leaves were vacuum infiltrated with 10⁸ or 10⁹ cfu/mL of the incompatible bacterium *Pseudomonas syringae* pv. *phaseolicola* (strain NPS 3121 kindly provided by Peter Lindgren, NC State University) or 10⁶ cfu/mL of the compatible bacterium *P. syringae* pv. *tabaci* (lab stock culture isolated from diseased tobacco in eastern NC). Bacteria were grown on King’s B solid medium (20 g peptone, 15 mL glycerol, 1.5 g K₂HPO₄, 1.5 g MgSO₄·6H₂O, 15 g agar, per Liter, pH 7.2) at 28 °C for 2 days, washed from the plates with sterile deionized H₂O, and the concentrations adjusted based on absorbance at 600 nm. For pyridoxine treatments, 100 mM pyridoxine was added to the bacterial solutions, and the pH was adjusted to match the pH of control suspensions (approximately pH 6.0). For northern analysis, three leaves per infiltration treatment (cut 1 in. above the petiole, each from a different plant) were collected, bulked, frozen in liquid nitrogen,

and stored at –80 °C. For assessing transcript regulation within and surrounding the HR tissue by quantitative polymerase chain reaction (qPCR), the lower sides of leaves were infiltrated with 10⁸ cfu/mL *P. syringae* pv. *phaseolicola* using a needleless syringe. Infiltrated regions were marked, and infiltrated tissue and the corresponding 5 mm surrounding region (staying within the lateral veins) were separately collected from three leaves, each from a different plant. Samples were collected at various time points, bulked, frozen in liquid nitrogen, and stored at –80 °C (method from Dorey et al., [22]).

2.6. DNA isolation and Southern analysis

Total genomic DNA was extracted using a 3% hexadecyltrimethylammonium bromide (CTAB) protocol. Ground leaf samples were incubated in CTAB extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris pH 8, 3% CTAB, and 1% β -mercaptoethanol) at 65 °C for 30 min, extracted twice with 24:1 (v:v) chloroform:isoamyl alcohol and precipitated with an equal volume of isopropanol. DNA was washed twice with 70% ethanol and resuspended in water. Samples were RNase treated (Promega, Madison, WI) using 1.5 μ L RNase per 300 μ L volume DNA at 37 °C for 15 min and stored at –20 °C. For Southern analysis, DNA (10 μ g) was digested with EcoRI and separated on a 1% gel. The gel was washed in 0.25 N HCl for 8 min, rinsed with deionized H₂O, then washed twice in 1.5 M NaCl/0.5 M NaOH while shaking for 15 min, and then washed twice in 0.5 M Tris pH 7.5/1.5 M NaCl while shaking for 15 min. After the washes, DNA was transferred onto a nylon membrane (Osmonics, Minnetonka, MN). Hybridization was performed at 65 °C using PerfectHyb Plus hybridization buffer (Sigma) and a digoxigenin-labeled DNA probe (Table 1) (Primers from Sigma-Genosys, The Woodlands, TX) according to manufacturer’s directions (Roche, Basel, Switzerland). The blot was washed under stringent conditions and developed following the digoxigenin-alkaline phosphatase kit (Roche) protocol and visualized on film.

2.7. RNA isolation and reverse transcription

Total RNA was extracted from leaf tissue using TRI Reagent (Sigma) following the manufacturer’s suggested protocol and adding a second phenol–chloroform extraction. RNA samples were resuspended in RNasefree (Ambion, Austin, TX) and DNase treated twice with DNA-free (Ambion) for 1 h. RNA was reverse transcribed in a ratio of 20 ng per 1 μ L using random hexamers and multiscribe reverse transcriptase as supplied in the Applied Biosystems Taqman RT-PCR Kit (Foster City, CA). Reverse transcription thermocycler parameters were 25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min.

2.8. Gene isolation of PDX1 and PDX2

Fragments of tobacco *PDX1* and *PDX2* were amplified from Burley 21 cDNA using degenerate primers (Table 1) based on sequence data available from *Arabidopsis*, rice, tomato, rubber tree, yeast, and *Cercospora* created by CODEHOP freeware

Table 1

Forward and reverse primers used for amplification of DNA from Burley 21 tobacco for gene isolation, probes for northern and southern analysis, and qPCR

Gene/experiment	Forward primer	Reverse primer
PDX1 degenerate ^a	5'-GCTGAGAGGTGGTGTATTATGGAYGTNGT-3'	5'-CATCATCATCCATATTTCTCAGAACTCK-DATRTCNCCC-3'
PDX2 degenerate ^a	5'-GACCAACATAAACCTACTTGGGGTACNT-GYGCNNGG-3'	5'-GATCAATAATTTCTTCAATAACAGGAGCNCKDA-TRAA-3'
PDX1 inverted ^b	5'-CGCACCAAGGGGGAAGC-3'	5'-CGGGGATGGTTACTGCCTGTT-3'
PDX2 inverted ^b	5'-GCCTCGATTGTACCGTCCACCGAAACT-3'	5'-TGAGGCCCGCACAAAGTACCCCAAGTAG-3'
PDX1 full length ^c	5'-ATGGCCGGAAGCGGTGTGGTAA-3'	5'-TCACTCAGAACGATTAGCAT-3'
PDX2 full length ^c	5'-ATGGTTGTGGGGTCTTGTCTTACAGG-GATCTTTCAAC-3'	5'-CTATTGGTATATGGGAA-3'
PDX1 probe ^d	5'-GCCCTTGAGCGCGTCCC-3'	5'-CATCATAAGCGCTGCGTCTGC-3'
PDX2 probe ^d	5'-CAGGAGCCCGGATAAAAAACAG-3'	5'-GTACTTGGCGAGGCCTCATT-3'
PR-1a probe ^d	5'-TGGGATTTGTCTCTTTTTCAC-3'	5'-TACCTGGAGGATCATAGTTGC-3'
PDX1 qPCR ^e	5'-CTTCGCGCCGACGATGAGAAC-3'	5'-TTACGGCAGCCACAGACAAA-3'
PDX2 qPCR ^e	5'-CAAATAAAGCAACTGGGCAGAA-3'	5'-CGGTGGACGGTACAATCGA-3'
PAL qPCR ^e	5'-GACGAGCTAAAGGCCGTGT-3'	5'-TGCAGGGTCCCCTTTCC-3'

^a Degenerate primers used to recover initial fragments of genes.

^b Inverted primers for recovery of full length sequence.

^c Full length primers for amplification of entire ORF.

^d Primers for labeling of probes for northern and Southern analysis.

^e Primers for qPCR.

(<http://bioinformatics.weizmann.ac.il/blocks/codehop.html>) [53]. A touchdown PCR program (94 °C for 5 min; 22 cycles of 94 °C for 30 s, 63 °C (−0.5 °C per cycle) for 40 s, 72 °C for 45 s; 20 cycles of 94 °C for 30 s, 52 °C for 40 s, 72 °C for 45 s) was used. Full length sequences were then amplified by inverted PCR for *PDX1* using the Genome Walker kit (BD Biosciences Clontech, Palo Alto, CA) with primers as listed in Table 1. For *PDX2*, full length sequence was amplified using inverted PCR on a tobacco cDNA library (Stratagene, La Jolla, CA). In this case, primers (Table 1) were paired with the T7 primer. For inverted PCR, primers designated in Table 1 as 'forward' amplify the 3' end of the gene and 'reverse' amplify the 5' end of the gene. Based on the inverted amplified sequences, primers were designed to isolate full length cDNA and genomic sequences for *PDX1* and *PDX2*. Primers were from Integrated DNA Technologies (IDT) (Coralville, IA). Sequences were analyzed using vector NTI (Informax of Invitrogen, Carlsbad, CA) and GenBank (NCBI) [2].

2.9. Quantitative PCR

Quantitative PCR (qPCR) experiments were performed on salicylic acid, methyl jasmonate, and pathogen-treated tissues. Samples were collected as described in Sections 2.4 and 2.5, and all samples were taken from previously unsampled plants. Gene-specific qPCR was performed using 5 µL cDNA mix (corresponding to 100 ng starting total RNA) in 25 µL total volume of reaction mix containing 2X SYBR Green mastermix (Applied Biosystems) and gene specific primers (0.8 pmol/µL final concentration) (Table 1) (Sigma-Genosys). For normalization, all genes were compared to expression of 18S ribosomal RNA (probe and primers from Applied Biosystems 18S kit) using Universal PCR master mix (Applied Biosystems). All reactions were done in triplicate. Real-time PCR reactions were carried out on an ABI7000 sequence detection

system or an MJ Research DNA Engine Opticon2 (parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C 1 min). For statistical analysis, 18S-normalized, untransformed data were compared using a paired *t*-test.

2.10. Northern analysis

Northern analysis was performed on salicylic acid and pathogen-treated tissue. Samples were collected as described in Sections 2.4 and 2.5, and all samples were taken from previously unsampled plants. Total RNA (20 ng) was separated on 1.2% formaldehyde gels and transferred onto nylon membranes (Osmonics). Hybridization was performed at 68 °C using PerfectHyb Plus hybridization buffer (Sigma) and digoxigenin-labeled DNA probes according to manufacturer's directions (Roche). Probes were generated from portions of *PDX1*, *PDX2* and *PR-1a* from tobacco (Table 1) (primers from Sigma-Genosys). Blots were washed under stringent conditions and developed following the digoxigenin-alkaline phosphatase kit (Roche) protocol and visualized on film. Ribosomal RNA visualized by ethidium bromide staining.

2.11. Bacterial growth curves

To assay the effect of pyridoxine on bacterial viability in vitro, 10⁸ and 10⁹ cells per ml of *P. syringae* pv. *phaseolicola* (grown on King's B solid medium for 2 days and suspended in sterile deionized H₂O) were incubated with or without 100 mM pyridoxine at room temperature. Samples were collected at 1 and 6 h, and serial dilutions plated on King's B medium and grown at 28 °C; colony counts were made at 48 h. For in planta growth assays, the terminal halves of tobacco leaves were infiltrated with 10⁸ or 10⁹ cfu/mL *P. syringae* pv. *phaseolicola* or 10⁶ cfu/mL *P. syringae* pv. *tabaci* with or without 100 mM pyridoxine. Leaf discs (each 2.54 cm²) were collected from one

leaf each on three separate plants per time point and bulked. All tissue was collected from previously unsampled plants. Leaf discs were surface sterilized in 10% bleach for 5 min followed by 70% ethanol for 10 min. Discs were ground in 5 mL of water and serial dilutions were plated on King's B medium and grown at 28 °C; colony counts were made at 48 h and the cfu/cm² leaf area determined. The in planta growth curve experiment was performed once.

2.12. Vitamin B₆ bioassay

Total vitamin B₆ was extracted from lyophilized tobacco leaf tissue using a modified protocol from Gregory [30]. Five plants were assayed individually for each treatment. After grinding lyophilized tissue in liquid nitrogen, 0.1 g tissue powder was mixed with 30 mL 0.44 N HCl and autoclaved at 121 °C for 4 h. The pH was brought to 4.8 using sodium acetate. To de-glycosylate and de-phosphorylate the vitamers, freshly prepared β-glycosidase (5 mg in water) and acid phosphatase (75 mg in water) were added to each flask, and flasks were placed in a 37 °C shaker at 70 rpm overnight. The mixture in each flask was then brought up to a 50 mL volume with deionized H₂O, filtered through miracloth, and filter sterilized using a 50 mL steriflip unit (0.22 μM) (Millipore, Bedford, MA). Extracts were stored in the dark at –20 °C until use.

Levels of total de-phosphorylated B₆ vitamers were determined using a bioassay that quantifies growth of a yeast (*S. cerevisiae*) strain (ATCC#9080) auxotrophic for vitamin B₆. This strain can utilize any of the vitamers (pyridoxine, pyridoxal, or pyridoxamine) for growth [30]. Cells were grown overnight at 30 °C with shaking at 220 rpm in 40 mL PYM medium (5.3 g/100 ml pyridoxine YM [Becton Dickinson, Franklin Lakes, NJ]). Pyridoxine YM obtained from the manufacturer does not contain pyridoxine and thus was amended, after autoclaving, with 40 ng pyridoxine. Overnight cultures were washed twice with PYM medium to remove residual pyridoxine. Assay tubes (14 mL) were set up to contain 2 × 10⁸ yeast cells in 4 mL of medium with 50 μL of leaf extract. Standards contained 0, 2, 4, 6, or 8 ng total pyridoxine per 4 mL volume. All samples and standards were run in triplicate. Tubes were incubated for 16 h at 30 °C/220 rpm. Growth was measured using a Beckman DU 650 spectrophotometer at OD₅₄₀. The vitamin B₆ content of the samples was determined based on comparison to the standard curve. Data are shown as the mean and standard error of the treatments. Statistical differences were determined using a *t*-test.

3. Results

3.1. Vitamin B₆ quenches superoxide and has antioxidant activity

Previous studies addressing the ability of B₆ vitamers to quench superoxide were limited to pyridoxine and were performed in complex systems, such as glucose-treated blood. We used a more direct chemical assay to quantify superoxide quenching, a colorimetric assay monitoring

inhibition of the reduction of cytochrome *c* by superoxide generated through xanthine and xanthine oxidase (Fig. 1). All three vitamers showed significant quenching abilities at the concentrations tested. Pyridoxal showed the strongest quenching activity, with 1 mM pyridoxal having equivalent quenching ability to one unit superoxide dismutase (SOD).

The antioxidant activity of B₆ vitamers was tested by assessing their ability to prevent lipid peroxidation in an assay measuring the coupled oxidation of β-carotene and linoleic acid (Fig. 2). All three vitamers showed significant antioxidant activity at concentrations of 40 μM and higher, with up to 80% prevention of β-carotene breakdown at a concentration of 4 mM. BHT (butylated hydroxytoluene), a strong phenolic antioxidant, was used as a control for antioxidant activity. Differences between BHT and the B₆ vitamers are magnified by the experimental system as BHT is more soluble in the liposomes than the water-soluble B₆ vitamers, thus increasing its effectiveness.

3.2. Isolation of *PDX1* and *PDX2* genes from tobacco

The vitamin B₆ biosynthetic genes, *PDX1* and *PDX2*, were identified in Burley 21 tobacco using a degenerate primer strategy based on sequences from plant and fungal homologues. Southern analysis suggested the presence of one copy each of *PDX1* and *PDX2* (data not shown). Degenerate primers were designed and used to amplify cDNA fragments of both genes. Using an inverted PCR protocol described in Section 2, full-length sequences for *PDX1* and *PDX2* were recovered.

Sequences for the tobacco *PDX1* and *PDX2* are available through GenBank (Accession numbers AY532656, AY532657, and AY532658). We reproducibly identified two distinct sequences for *PDX1*; these are hypothesized to be either two separate alleles or two separate copies of *PDX1* in tobacco. The two sequences, both 930 nucleotides in length, differ by 15 nucleotides, but the predicted amino acid sequence is unchanged. *PDX1* homologues in general are highly conserved proteins; the tobacco homologues show 80–90% identity at the amino acid level with homologues from *Arabidopsis*, rice, bean, and rubber tree. As is common for all homologues identified to date, the tobacco *PDX1* homologues are composed of one exon. *PDX2* in tobacco is a smaller coding sequence of 756 nucleotides and contains introns. It is less conserved than *PDX1*, with 70–80% identity at the amino acid level with identified homologues in *Arabidopsis*, rice and maize.

3.3. Regulation of tobacco vitamin B₆ biosynthetic genes during salicylic acid and methyl jasmonate treatment

Transcript abundance was measured by northern analysis and qPCR, with the exception of *PDX2*, which proved difficult to assess by northern analysis due to its low expression level and thus was measured using qPCR only. Transcript abundance in response to salicylic acid and methyl jasmonate treatment is shown in Figs. 3 and 4. The two versions of *PDX1* were measured in total as we did not differentiate between the versions through northern analysis or qPCR. Leaf tissue of tobacco plants sprayed with 2 or 5 mM salicylic acid showed

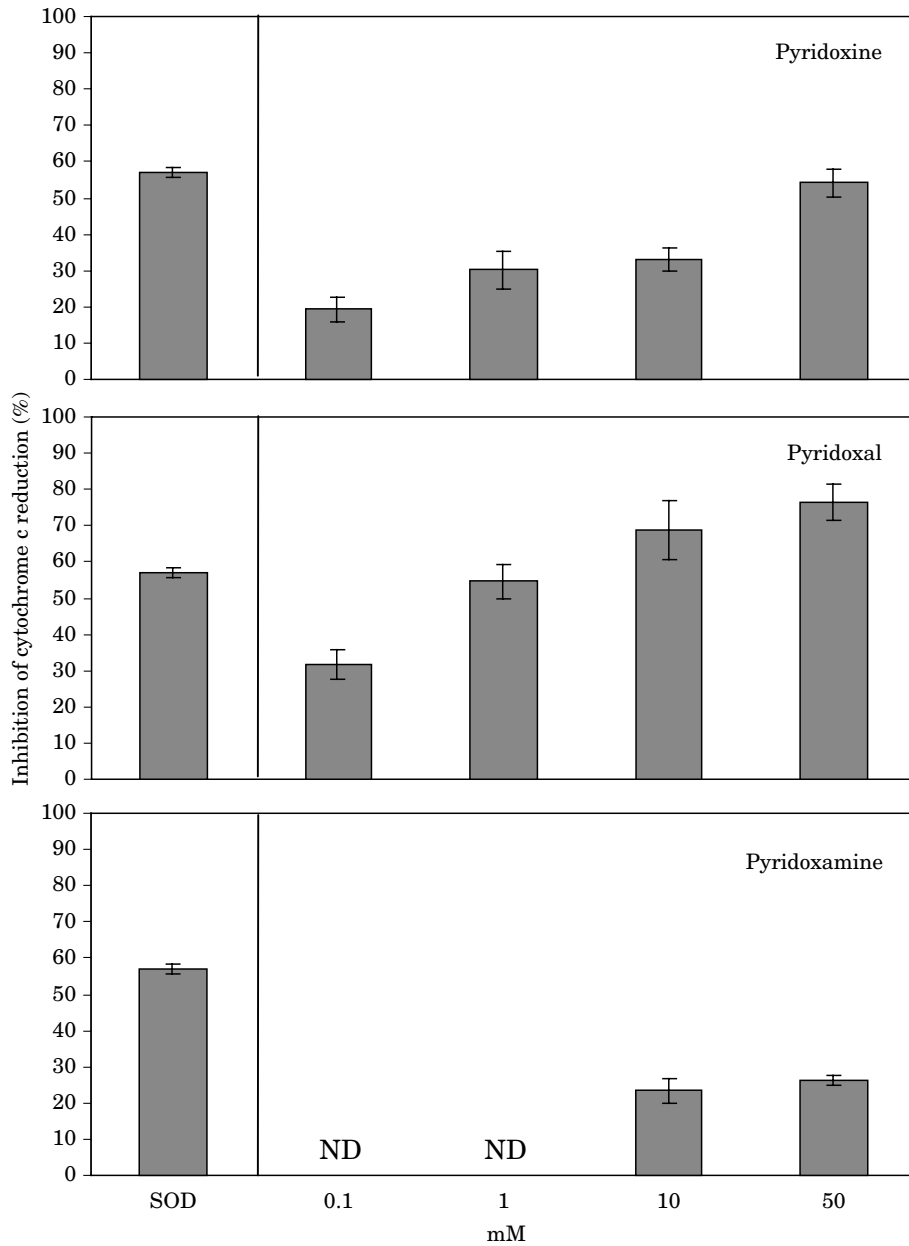


Fig. 1. Quenching of superoxide by B₆ vitamins pyridoxine, pyridoxal, and pyridoxamine, measured as the percent inhibition of superoxide-mediated cytochrome *c* reduction. Superoxide dismutase (SOD) control represents one unit SOD. Error bars represent standard error of three replicates. Quenching of superoxide by all three vitamins was statistically different from the water control at all concentrations tested ($P < 0.05$). ND = not done.

a dose-dependent increase in *PDX1* transcript abundance 6 h after treatment, followed by a decline at 24 and 48 h (Fig. 3). Shown as a control for salicylic acid response, *PR-1a* transcript levels began increasing at 6 h and continued to increase through 48 h. Results for *PDX1* were confirmed by qPCR; in two separate experiments, *PDX1* transcript reached an average of three-fold (2 mM) and 4.3-fold (5 mM) increase over the water control at 6 h, and then declined, reaching control expression levels or lower, by 24 h (Fig. 4). *PDX2* transcript abundance, by contrast, remained at or below control levels for all time points tested. Tobacco plants sprayed with 1 mM methyl jasmonate showed increased *PDX1* and *PDX2* transcript abundance at 24 h, dropping back to control levels by 48 h (Fig. 4).

3.4. Regulation of tobacco vitamin B₆ biosynthetic genes during pathogen response

Using northern analysis (*PDX1* only) and qPCR (*PDX1* and *PDX2*), we assessed *PDX1* and *PDX2* transcript abundance in tobacco during the defense response to *P. syringae* pv. *phaseolicola*, a pathogen that is compatible on bean, but incompatible on tobacco due to the induction of the hypersensitive response. We chose to use this pathogen, as *Pseudomonas* species contain the alternate vitamin B₆ biosynthetic pathway similar to *E. coli* and thus do not contain *PDX1* or *PDX2* homologues [48], ensuring that we were only measuring plant transcript. Tobacco leaves were vacuum-infiltrated or syringe-infiltrated

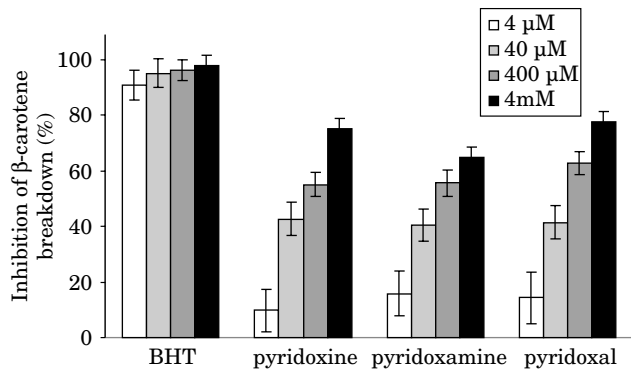


Fig. 2. Antioxidant activity of B₆ vitamers determined by measuring the inhibition of β -carotene breakdown caused by the coupled oxidation of β -carotene and linoleic acid. Butylated hydroxytoluene (BHT), a strong phenolic antioxidant, was used as a control for antioxidant activity. Error bars represent standard error of three replicates. Antioxidant activity of BHT was statistically different from the water control at all concentrations tested ($P < 0.05$). For each of the B₆ vitamers, antioxidant activity was statistically different from the water control at concentrations of 40 μ M and above ($P < 0.05$).

with 10^8 cfu/mL of the bacterium. The infiltrated regions showed chlorosis and bronzing at 24 h and necrosis by 48 h, symptoms indicative of the HR. Transcript accumulation was assessed in the infiltrated tissue and in the 5 mm region surrounding the infiltrated tissue (Fig. 5).

Through northern analysis, a temporary increase in *PDX1* transcript abundance was observed within both the pathogen-infiltrated and the water-infiltrated (control) tobacco leaf tissue (Fig. 6). A similar increase in transcript was also observed in infiltrations of tomato leaves with water (data not shown), suggesting that *PDX1* may be responsive to wounding or general hypoxia stress caused by water infiltration. In tobacco, while the increase in *PDX1* transcript for water-infiltrated tissue continued from 1 to 8 h post-infiltration, *PDX1* transcript abundance in the pathogen-infiltrated samples fell sharply after 4 h post-infiltration. Quantitative PCR data measuring the fold change in transcript from pathogen-treated leaves over water

control-treated leaves confirmed the northern results (Fig. 7(a)). *PDX1* transcript levels were almost identical for the water-infiltrated and pathogen-infiltrated tissues soon after infiltration, with pathogen-infiltrated tissue showing an average 1.16-fold increase in transcript abundance relative to the water-infiltrated tissue 2 h post-infiltration. By 6 and 10 h post-infiltration, transcript abundance of *PDX1* in pathogen-treated leaves dropped to half of that of the water controls. Although this drop confirms the northern results, statistical analysis of the qPCR data did not show a significant difference at $P < 0.05$ (6 h, $P = 0.108$; 10 h, $P = 0.065$).

As with *PDX1*, *PDX2* transcript was similar to control levels at 2 h post infiltration, and then dropped at 6 and 10 h (Fig. 7(b)). The decrease at both time points was statistically significant (6 h, $P = 0.04$; 10 h, $P = 0.003$).

To ensure that the drop in transcript observed for *PDX1* and *PDX2* in tissue undergoing the HR was not due to a general inability to transcribe genes, we also measured the abundance of phenylalanine ammonia lyase (*PAL*) transcript by qPCR. At all time points tested, *PAL* transcript levels were higher in tissue undergoing an HR than in water-treated tissue, reaching a 41-fold increase over the water control at 10 h post-infiltration (Fig. 7(c)). These results show that in tissue undergoing an HR, the decrease in transcript abundance of B₆ biosynthetic genes is not simply due to the cells' inability to transcribe genes during an HR.

Transcript abundance was also measured in tissues in a 5 mm region bordering the infiltrated region (Fig. 8). When averaged over four experiments, the trend in transcript abundance shows that *PDX1* transcript levels stay at or slightly above water control levels, reaching a high of 1.8- and 1.9-fold increase over water control at 10 and 24 h post-infiltration, respectively (Fig. 8(a)). The difference observed at 10 h was statistically significant ($P = 0.023$). *PDX2* transcript levels did not differ significantly from water-control values at any time point (Fig. 8(b)). *PAL* transcript abundance increased dramatically in the 5 mm border region, reaching maximum levels (17.5-fold increase over water control) at 10 h post-infiltration (Fig. 8(c)).

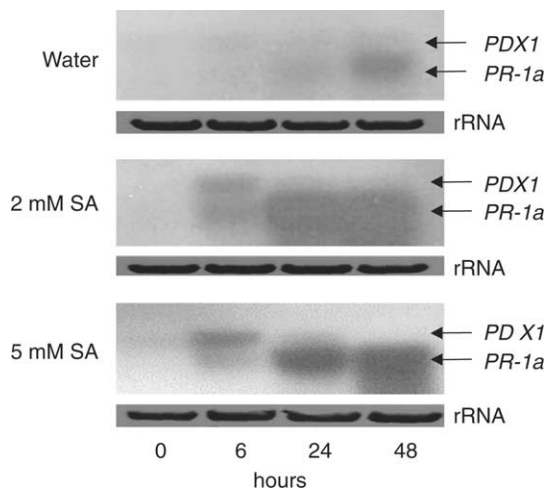


Fig. 3. Northern analysis of tobacco leaf tissue from plants sprayed with 2 or 5 mM salicylic acid or water. Blots probed with digoxigenin-dUTP labeled *PDX1* and *Pr-1a* (included as an SAR control). 25S rRNA is shown below blots as a gel loading control.

3.5. Vitamin B₆ interferes with the plant-pathogen defense response

As vitamin B₆ biosynthesis is both up- and down-regulated during plant defense responses, we wished to test the effect of modified levels of vitamin B₆ on a plant's response to both compatible and incompatible pathogens. We initially attempted to increase the levels of vitamin B₆ in tobacco by constitutive expression of fungal homologues of *PDX1* and *PDX2*, however, these efforts were unsuccessful (Herrero and Daub, unpublished). We thus opted to temporarily increase pyridoxine levels in leaf tissue through co-infiltration along with the *Pseudomonas* inoculum.

The effect of infiltration of leaves with pyridoxine on the concentration of pyridoxine in the infiltrated tissue was assayed using a yeast bioassay. Total B₆ content in infiltrated leaves was determined by extracting tissue, dephosphorylating the vitamers by acid hydrolysis and phosphatase treatment, and

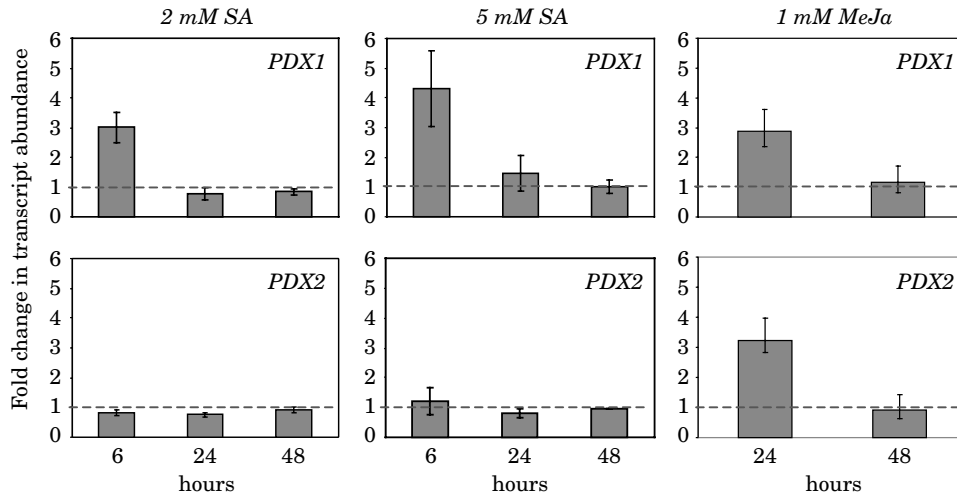


Fig. 4. Transcript abundance of *PDX1* and *PDX2* in tobacco leaf tissue sprayed with 2 or 5 mM salicylic acid or 1 mM methyl jasmonate measured by qPCR and normalized to 18S expression. Salicylic acid data are shown as the average fold change of transcript from salicylic acid-treated over water control-treated plants from two experiments. Methyl jasmonate data show the average of three qPCR replicates from one experiment. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. Error bars=experimental values (salicylic acid) or standard error (methyl jasmonate).



Fig. 5. (a) Front and (b and c) back of a tobacco leaf showing a hypersensitive response after needleless syringe infiltration of the incompatible bacterium *Pseudomonas syringae* pv. *phaseolicola*. Infiltrated region was outlined with a sharpie marker on the backs of leaves. (c) The infiltrated regions and the 5 mm surrounding regions were tested separately, staying within major lateral veins, for transcript abundance.

then assaying the amount by quantifying growth of a yeast pyridoxine auxotroph; we have found this assay to be more sensitive than HPLC for determination of total vitamin B₆ content. Leaves were infiltrated and assayed immediately upon disappearance of the water-soaking symptoms (2 h). Tobacco leaf tissue infiltrated with 100 mM pyridoxine contained an average 185 (+/- 8.1) nmoles pyridoxine per gram dry weight as compared to 130 (+/- 17.1) nmoles pyridoxine per gram leaf tissue for the water-infiltrated control. This difference was statistically significant ($P=0.037$). Thus, infiltration of leaf tissue with 100 mM pyridoxine significantly increased the concentration of pyridoxine in the leaf tissue, but still within physiologically acceptable levels. Possible toxicity of 100 mM pyridoxine toward *P. syringae* pv. *phaseolicola* was also tested. Bacteria were mixed with 100 mM pyridoxine and tested for viability at 1 and 6 h. The pyridoxine treatment had little effect (11% reduction compared to water treatment at 6 h) on survival in vitro.

Tobacco plants were co-infiltrated with 100 mM pyridoxine mixed with bacterial inoculum. Pyridoxine treatment led to a delayed hypersensitive response in the incompatible interaction (Fig. 9). In the treatment with bacteria alone, symptoms of the HR (tissue collapse followed by necrosis) were first visible at 10 h. In the treatment with bacteria plus pyridoxine, there were no symptoms at 10 h, and only limited tissue wilting

at 24 h. Some hypersensitive necrosis developed by 72 h, but it was less extensive than in the treatment without pyridoxine. In treatments with pyridoxine alone, there were no visible symptoms at 10 and 24 h, but by 72 h, some leaves showed slight flecking. This damage was clearly distinguishable from symptoms seen with the bacterial treatments. An in vivo bacterial growth curve experiment showed no differences in bacterial populations in tissues with and without pyridoxine, suggesting that the delay in the HR was not due to an effect on bacterial numbers (data not shown).

Tobacco plants infiltrated with the compatible bacterium *P. syringae* pv. *tabaci* plus pyridoxine showed increased

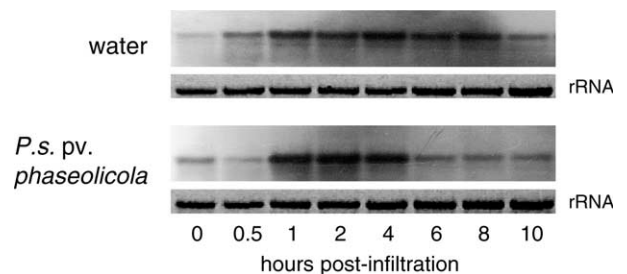


Fig. 6. Northern analysis assessing *PDX1* transcript abundance following vacuum infiltration of tobacco leaves with 10^8 cfu/ml of the incompatible bacterium *P. syringae* pv. *phaseolicola* or water control. 25S rRNA shown below blots as loading control.

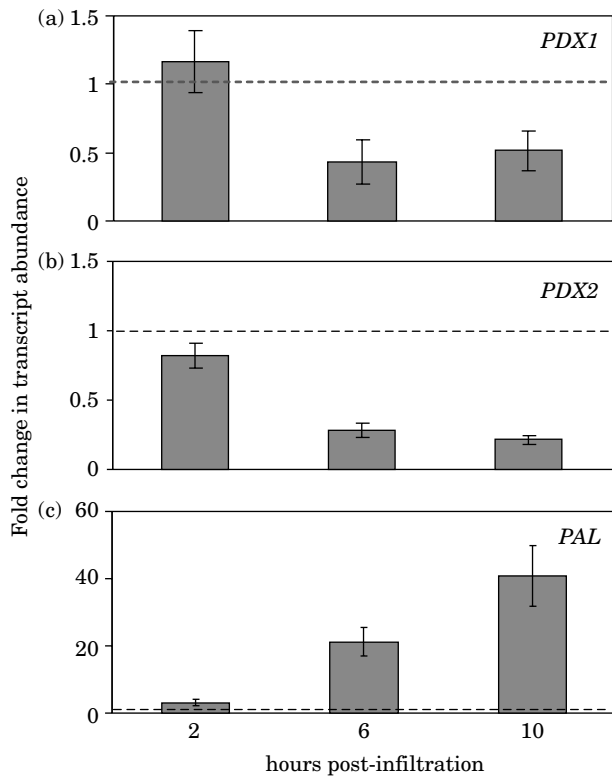


Fig. 7. Fold change in transcript abundance of (a) *PDX1*, (b) *PDX2*, and (c) *PAL* in tobacco leaf tissue infiltrated with 10^8 cfu/mL of the incompatible bacterium *P. syringae* pv. *phaseolicola*. Transcript abundance was measured by qPCR and normalized to 18S expression. Data are shown as the average fold change of transcript from pathogen-treated plants over water control-treated plants from four experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. Error bars = standard error.

disease symptoms compared to those infiltrated with bacteria alone (Fig. 10). At 96 h, the pyridoxine-treated plants had stronger chlorosis with more areas of necrosis. The minor flecking seen on some leaves infiltrated with pyridoxine alone was unchanged from that seen at 72 h. As with the incompatible interaction, an *in vivo* bacterial growth curve showed no correlation with symptom expression, again suggesting that the effect of pyridoxine on disease symptoms was not due to an effect on bacterial numbers (data not shown).

4. Discussion

Previous studies in animal systems have connected pyridoxine with protection against oxidative damage [35–37, 40,57]. We confirmed these studies and tested the B₆ vitamers pyridoxine, pyridoxal and pyridoxamine with controlled *in vitro* assays. Our lab has previously shown that B₆ vitamers are potent quenchers of singlet oxygen [7]. The work described here confirms that B₆ vitamers have antioxidant activity and are also potent quenchers of another species of active oxygen, superoxide.

Our data show that pyridoxine acts as an antioxidant in planta by interfering with plant defense mechanisms, a process involving active oxygen production. One of the earliest responses to pathogen attack is the production of superoxide,

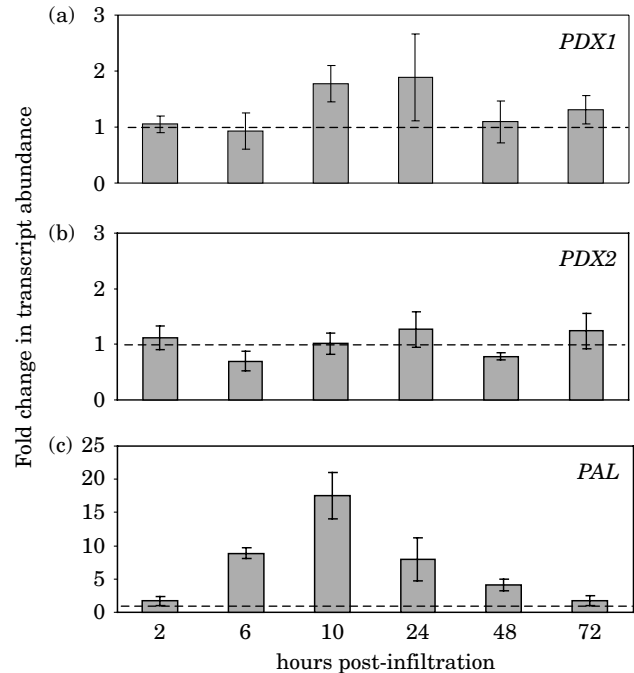


Fig. 8. Transcript abundance of (a) *PDX1*, (b) *PDX2*, and (c) *PAL* in 5 mm tobacco leaf tissue surrounding the region infiltrated with the incompatible bacterium *P. syringae* pv. *phaseolicola*. Transcript abundance was measured by qPCR and normalized to 18S expression. Data are shown as the average fold change of transcript from pathogen-treated plants over water control-treated plants from four experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. Error bars = standard error.

generated through an NADPH-dependent pump in the cell membrane [9,17,20,38,58,59]. During a defense response, both to compatible and incompatible pathogens, plants rely on producing active oxygen species for numerous defense mechanisms including direct antimicrobial activity, as substrates in cell wall fortifications, and as signaling molecules for the activation of defense pathways [5,8,15,20,29,44,45,47]. When pyridoxine levels were increased in leaf tissue by infiltration, we observed a delay in the hypersensitive response during exposure to an incompatible pathogen and a pronounced increase in disease symptoms during exposure to a compatible pathogen. We hypothesize that defense—related changes, caused by increased production of AOS, were hindered by pyridoxine, supporting a hypothesis that B₆ vitamers can act as antioxidants in plants and may act as important modulators of redox status during pathogen defense responses.

Congruent with pyridoxine interfering with a pathogen defense response, our regulation studies show that *de novo* vitamin B₆ biosynthetic genes, *PDX1* and *PDX2*, are regulated in a manner consistent with decreasing antioxidants in tissues mounting a pathogen response, but maintaining antioxidant levels in tissues bordering a defense response. In leaf tissue treated with an incompatible pathogen leading to an HR, we saw a sharp decrease in transcript abundance for these genes, in line with the plant's goal of decreasing antioxidants in tissue requiring AOS for mounting a defense response. This decrease is not due to a general inability of a cell to transcribe genes

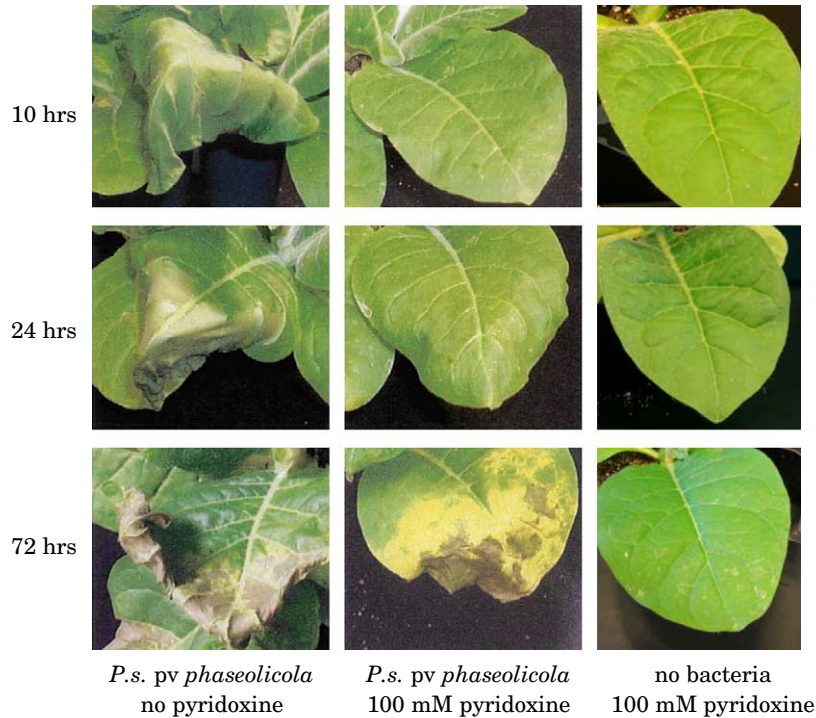


Fig. 9. Response in tobacco plants infiltrated with 10^9 cfu/ml of the incompatible bacterium *P. syringae* pv. *phaseolicola* only, 10^9 cfu/ml *P. syringae* pv. *phaseolicola* with 100 mM pyridoxine, or 100 mM pyridoxine only. Infiltration of inoculum with pyridoxine delayed and decreased the collapse and necrosis associated with the HR.

during an HR, as we saw large increases in transcript for *PAL*, a gene known to accumulate in HR tissue and which encodes a key enzyme in secondary metabolism pathways leading to the production of many defense compounds [22]. These results are consistent with the HR as a programmed cell death relying on active production of proteins, and supports our hypothesis that the decrease in transcript abundance of B₆ biosynthetic genes in tissue undergoing an HR is not simply due to the cells' inability to transcribe genes during an HR, but is likely due to an active process to decrease B₆ amounts. In untreated tissue surrounding a developing HR, we saw no change from control except for a slight increase in *PDX1* transcript 10 and 24 h post-infiltration, suggestive of a need for vitamin B₆ in the surrounding tissue, perhaps involved in protection from AOS damage. And, in leaf tissue treated with salicylic acid or methyl jasmonate, defense compounds produced both in cells directly affected by

pathogens and in cells from surrounding regions, a transient increase was observed for transcripts of *PDX1* (both treatments) and *PDX2* (methyl jasmonate only).

The results we found for *PDX1* and *PDX2* transcript accumulation mesh well with the limited data published on the activities and levels of other AOS scavengers during plant defense. For example, ascorbate peroxidase and catalase activity were suppressed in cells directly affected by an incompatible pathogen and undergoing an HR [23,39,49], and cultured tobacco cells treated with NO and H₂O₂ generators had decreased ascorbate peroxidase activity and decreased pools of ascorbate and glutathione during the HR [19]. Further, transgenic plants that overproduce catalase showed a decreased ability to mount a defense against pathogen attack [49,51]. These studies reflect the necessity of reducing antioxidant ability in order to mount a successful defense response.

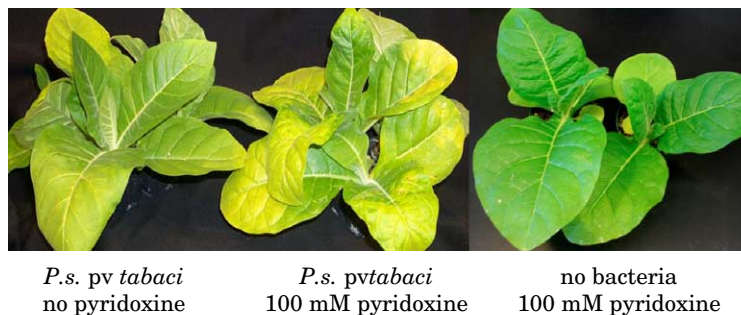


Fig. 10. Symptoms on tobacco plants at 4 days following infiltration with 10^6 cfu/ml of the compatible bacterium *P. syringae* pv. *tabaci* (left) as compared to symptoms when bacteria were co-infiltrated with 100 mM pyridoxine (middle). Pyridoxine-only control is shown on the right. Infiltration of inoculum with pyridoxine increased the severity of chlorotic and necrotic symptoms associated with disease.

In all of our infiltration experiments, including water controls, we observed an increase in transcript accumulation for *PDX1* approximately 2–6 h post-treatment. We hypothesize that this is a response to water stress or wounding. A recent study showed that a *PDX1* homologue from bean (*Phaseolus vulgaris*) showed increased transcript abundance in response to wounding [28]. Recent data from our lab on gene regulation in *Arabidopsis* suggest that B₆ de novo biosynthetic genes are also highly regulated by light cycles and abiotic stress conditions. These results, combined with the studies from *Arabidopsis* showing that vitamin B₆ is needed for salt tolerance and that excess B₆ can reduce singlet oxygen-induced death during light treatment of flu mutants [14,55], support a role for vitamin B₆ in protection against other AOS-producing scenarios.

The vitamin B₆ pathway in plants has only recently been discovered (25). In this study, we identified the homologues for *PDX1* and *PDX2* in Burley 21 tobacco. We identified two sequences for *PDX1*, differing by 15 nucleotides with no difference in predicted amino acid sequence. These may represent two alleles, or more likely, two separate copies in the allotetraploid tobacco. *Arabidopsis* and rice each contain three full length copies of *PDX1* and rubber tree has two copies, however, only one copy has been identified in bean. We identified only one copy of *PDX2* in Burley 21 tobacco, which is true also for rice and *Arabidopsis*. Our regulation data suggest that *PDX2* is not as tightly controlled during the defense response as *PDX1*, consistent with a conclusion that *PDX1* is the more important and highly regulated protein as it carries out the final biosynthetic reaction using a substrate produced by *PDX2*.

Interestingly, the tobacco *PDX1* genes have no introns, a conserved phenomenon with all identified homologues to date. Lack of introns is characteristic of some genes encoding stress-responsive proteins because proteins involved in splicing are affected by stress [62]. Due to altered splicing mechanisms, some genes are differentially spliced under varied environmental stimuli [1,61]. One example of this affect on splicing in plants is an invertase gene from potato that shows an altered splicing pattern during cold treatment [10].

In summary, we have demonstrated that vitamin B₆ biosynthetic genes are regulated during plant defense responses in a manner consistent with this vitamin's activity as an antioxidant and quencher of superoxide. Further, increasing the levels of pyridoxine delays defense responses, reducing and delaying the hypersensitive response and increasing severity of disease symptoms. Thus, our study demonstrates that in addition to its critical role as a cofactor for enzymes involved in growth and metabolism, vitamin B₆ is also a strong antioxidant with potential importance during the plant-pathogen defense response.

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References

- [1] Akker SA, Smith PJ, Chew SL. Nuclear post-transcriptional control of gene expression. *J Mol Endocrinol* 2001;27:123–31.
- [2] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–10.
- [3] Antelmann H, Bernhardt J, Schmid R, Mach H, Volker U, Hecker M. First steps from a two dimensional protein index towards a response-regulation map for *Bacillus subtilis*. *Electrophoresis* 1997;18:1451–63.
- [4] Arps PJ, Winkler ME. An unusual genetic link between vitamin B₆ biosynthesis and tRNA pseudouridine modification in *Escherichia coli* K-12. *J Bacteriol* 1987;169:1071–9.
- [5] Baker JC, Orlandi EW. Active oxygen in plant pathogenesis. *Annu Rev Phytopathol* 1995;33:299–321.
- [6] Bauer JA, Bennett EM, Begley TP, Ealick SE. Three-dimensional structure of YaaE from *Bacillus subtilis*, a glutaminase implicated in pyridoxal-5'-phosphate biosynthesis. *J Biol Chem* 2004;279:2704–11.
- [7] Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF. Vitamin B₆ (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochem Photobiol* 2000;71:129–34.
- [8] Bolwell GP. Role of active oxygen species and NO in plant defence responses. *Curr Opin Plant Biol* 1999;2:287–94.
- [9] Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, et al. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *J Exp Bot* 2002;53:1367–76.
- [10] Bournay A, Hedley P, Maddison A, Waugh R, Machray G. Exon skipping induced by cold stress in a potato invertase gene transcript. *Nucleic Acids Res* 1996;24:2347–51.
- [11] Braun EL, Fuge EK, Padilla PA, Werner-Washburne M. A stationary-phase gene in *Saccharomyces cerevisiae* is a member of a novel, highly conserved gene family. *J Bacteriol* 1996;178:6865–72.
- [12] Brosche M, Schuler MA, Kalbina I, Connor L, Strid A. Gene regulation by low level UV-B radiation: identification by DNA array analysis. *Photochem Photobiol Sci* 2002;1:656–64.
- [13] Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, et al. Global transcriptional responses of fission yeast to environmental stress. *Mol Biol Cell* 2003;14:214–29.
- [14] Danon A, Miersch O, Felix G, op den Camp RGL, Apel K. Concurrent activation of cell death-regulating signaling pathways by singlet oxygen in *Arabidopsis thaliana*. *Plant J* 2005;41:68–80.
- [15] Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Breusegem F. Dual action of the active oxygen species during plant stress responses. *Cell Mol Life Sci* 2000;57:779–95.
- [16] Daub ME. Resistance of fungi to the photosensitizing toxin, cercosporin. *Phytopathology* 1987;77:1515–20.
- [17] De Gara L, de Pinto M, Tommasi F. The antioxidant systems vis-a-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiol Biochem* 2003;41:863–70.
- [18] Dempsey WB. Characterization of pyridoxine auxotrophs of *Escherichia coli*: results of P1 transduction. *J Bacteriol* 1969;97:1403–10.
- [19] de Pinto MC, Tommasi F, De Gara L. Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco bright-yellow 2 cells. *Plant Physiol* 2002;130:698–708.
- [20] Doke N. Generation of superoxide anion by potato-tuber protoplasts during the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. *Physiol Plant Pathol* 1983;23:359–67.
- [21] Dong Y-X, Sueda S, Nikawa J-I, Kondo H. Characterization of the products of the genes *SNO1* and *SNZ1* involved in pyridoxine synthesis in *Saccharomyces cerevisiae*. *Eur J Biochem* 2004;271:745–52.
- [22] Dorey S, Baillieux F, Pierrel M-A, Saindrenan P, Fritig B, Kauffmann S. Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein. *Mol Plant Microbe Interact* 1997;10:646–55.

- [23] Dorey S, Baillieux F, Saindrenan P, Fritig B, Kauffmann S. Tobacco class I and class II catalases are differentially expressed during elicitor-induced hypersensitive cell death and localized acquired resistance. *Mol Plant Microbe Interact* 1998;11:1102–9.
- [24] Drewke C, Leistner E. Biosynthesis of vitamin B₆ and structurally related derivatives. In: Litwack G, Begley T, editors. *Vitamins and hormones. Advances in research and applications*. San Diego: Academic Press; 2001. p. 121–55.
- [25] Ehrenshaft M, Bilski P, Li MY, Chignell CF, Daub ME. A highly conserved sequence is a novel gene involved in de novo vitamin B₆ biosynthesis. *Proc Natl Acad Sci USA* 1999;96:9374–8.
- [26] Ehrenshaft M, Daub ME. Isolation of *PDX2*, a second novel gene in the pyridoxine biosynthesis pathway of eukaryotes, archaeobacteria, and a subset of eubacteria. *J Bacteriol* 2002;183:3383–90.
- [27] Ehrenshaft M, Jenns AE, Chung KR, Daub ME. *SORI*, a gene required for photosensitizer and singlet oxygen resistance in *Cercospora* fungi is highly conserved in divergent organisms. *Mol Cell* 1998;1:603–9.
- [28] Graham CM, Ehrenshaft E, Hausner G, Reid DM. A highly conserved gene for vitamin B₆ biosynthesis may have consequences for stress and hormone responses in plants. *Physiol Plant* 2004;121:8–14.
- [29] Grant JJ, Loake GJ. Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol* 2000;124:21–9.
- [30] Gregory JF. Relative activity of the nonphosphorylated B-6 for *Saccharomyces uvarum* and *Kloeckera brevis* in vitamin B-6 microbiological assays. *J Nutr* 1982;112:1643–7.
- [31] Hammerschmidt PA, Pratt DE. Phenolic antioxidants of dried soybeans. *J Food Sci* 1978;43:556–9.
- [32] Hill RE, Spenser ID. Biosynthesis of vitamin B₆. In: Dolphin D, Poulson R, Avramovic O, editors. *Coenzymes and cofactors*, vol. 1. NY: Wiley; 1986. p. 417–76.
- [33] Hill RE, Spenser ID. Biosynthesis of vitamin B₆. In: Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Schaechter M, Umberger HE, editors. *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*, vol. 2. Washington, DC: ASM Press; 1996. p. 695–703.
- [34] Hockney RC, Scott TA. The isolation and characterization of three types of vitamin B₆ auxotrophs of *Escherichia coli* K12. *J Gen Microbiol* 1979;110:275–83.
- [35] Jain SK, Lim G. Pyridoxine and pyridoxamine inhibit superoxide radicals and prevent lipid peroxidation, protein glycosylation, and (Na⁺ + K⁺)-ATPase activity reduction in high glucose-treated human erythrocytes. *Free Radic Biol Med* 2001;30:232–7.
- [36] Jain AK, Lim G, Langford M, Jain SK. Effect of high-glucose levels on protein oxidation in cultured lens cells, and in crystalline and albumin solution and its inhibition by vitamin B₆ and *N*-acetylcysteine: its possible relevance to cataract formation in diabetes. *Free Radic Biol Med* 2002;33:1615–21.
- [37] Kannan K, Jain SK. Effect of vitamin B₆ on oxygen radicals, mitochondrial membrane potential, and lipid peroxidation in H₂O₂-treated U937 monocytes. *Free Radic Biol Med* 2004;36:423–8.
- [38] Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C. A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca²⁺ binding motifs. *Plant Cell* 1998;10:255–66.
- [39] Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, et al. Nitric oxide and salicylic acid signaling in plant defense. *Proc Natl Acad Sci USA* 2000;97:8849–55.
- [40] Lakshmi R, Lakshmi AV, Divan PV, Bamji MS. Effect of riboflavin or pyridoxine deficiency on inflammatory response. *Indian J Biochem Biophys* 1991;28:481–4.
- [41] Lam HM, Tancula E, Dempsey WB, Winkler ME. Suppression of insertions in the complex *pdjX* operon of *Escherichia coli* K-12 by *lon* and other mutations. *J Bacteriol* 1992;174:1554–67.
- [42] Lam HM, Winkler ME. Characterization of the complex *pdxH*-*tyrS* operon of *Escherichia coli* K-12 and pleiotropic phenotypes caused by *pdxH* insertion mutations. *J Bacteriol* 1992;174:6033–45.
- [43] Lam HM, Winkler ME. Metabolic relationships between pyridoxine (vitamin B₆) and serine biosynthesis in *Escherichia coli* K-12. *J Bacteriol* 1990;172:6518–28.
- [44] Lamb C, Dixon RA. The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 1997;48:251–75.
- [45] Low PS, Merida JR. The oxidative burst in plant defense: function and signal transduction. *Physiol Plant* 1996;96:533–42.
- [46] Man TK, Zhao G, Winkler ME. Isolation of a *pdjX* point mutation that bypasses the requirement for the *PdxH* oxidase in pyridoxal-5-phosphate coenzyme biosynthesis in *Escherichia coli* K-12. *J Bacteriol* 1996;178:2445–9.
- [47] Mehdy MC, Sharma YK, Sathasivan K, Bays NW. The role of activated oxygen species in plant disease resistance. *Physiol Plant* 1996;98:365–74.
- [48] Mittenhuber G. Phylogenetic analyses and comparative genomics of vitamin B₆ (pyridoxine) and pyridoxal phosphate biosynthesis pathways. *J Mol Microbiol Biotechnol* 2001;3:1–20.
- [49] Mittler R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 2002;7:405–10.
- [50] Padilla PA, Fuge EK, Crawford ME, Errett A, Werner-Washburne M. The highly conserved, coregulated *SNO* and *SNZ* gene families in *Saccharomyces cerevisiae* respond to nutrient limitation. *J Bacteriol* 1998;180:5718–26.
- [51] Polidoros AN, Mylona PV, Scandalios JG. Transgenic tobacco plants expressing the maize *Cat2* gene have altered catalase levels that affect plant–pathogen interactions and resistance to oxidative stress. *Transgenic Res* 2001;10:555–69.
- [52] Roa BB, Connolly DM, Winkler ME. Overlap between *pdxA* and *ksgA* in the complex *pdxA*-*ksgA*-*apaG*-*apaH* operon of *Escherichia coli* K-12. *J Bacteriol* 1989;171:4767–77.
- [53] Rose TM, Henikoff JG, Henikoff S. CODEHOP (CONsensus-DEgenerate hybrid oligonucleotide primer) PCR primer design. *Nucleic Acids Res* 1998;31:3763–6.
- [54] Schoenlein PV, Roa BB, Winkler ME. Divergent transcription of *pdxB* and homology between the *pdxB* and *serA* gene products in *Escherichia coli* K-12. *J Bacteriol* 1989;171:6084–92.
- [55] Shi HZ, Xiong LM, Stevenson B, Lu TG, Zhu JK. The *Arabidopsis* salt overly sensitive 4 mutants uncover a critical role for vitamin B₆ in plant salt stress tolerance. *Plant Cell* 2002;14:575–88.
- [56] Sivasubramanian S, Vanniashingham VM, Tan CT, Chua NH. Characterization of HEVER, a novel stress-induced gene from *Hevea brasiliensis*. *Plant Mol Biol* 1995;29:173–8.
- [57] Stocker P, Lesgards J-F, Vidal N, Chaliel F, Prost M. ESR study of a biological assay on whole blood: antioxidant efficiency of various vitamins. *Biochim Biophys Acta* 2003;1621:1–8.
- [58] Torres MA, Dangl JL, Jones JDG. *Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci USA* 2002;99:517–22.
- [59] Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG. Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*gp91^{phox}*). *Plant J* 1998;14:365–70.
- [60] Wetzelschloffer DK, Ehrenshaft M, Denslow SA, Daub ME. Functional complementation between the *PDX1* vitamin B₆ biosynthetic gene of *Cercospora nicotianae* and *pdjX* of *Escherichia coli*. *Fed Eur Biochem Soc Lett* 2004;564:143–6.
- [61] Wilson KF, Cerione RA. Signal transduction and post-transcriptional gene expression. *Biol Chem* 2000;381:357–65.
- [62] Yost HJ, Lindquist S. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* 1986;45:185–93.
- [63] Zhao G, Winkler ME. 4-Phospho-hydroxy-L-threonine is an obligatory intermediate in pyridoxal 5'-phosphate coenzyme biosynthesis in *Escherichia coli* K-12. *Fed Eur Microbiol Soc Microbiol Lett* 1996;135:275–80.