## ABSTRACT

DENSLOW, SHERI ANN. The Function and Regulation of $B_6$ Biosynthetic Genes by Oxidative Stress in Plants. (Under the direction of Dr. Margaret E. Daub and Dr. Arthur Weissinger)

Vitamin $B_6$ has consistently been associated in diverse organisms with conditions of cellular oxidative stress. Plants contain an alternate, and previously uncharacterized pathway for vitamin $B_6$ biosynthesis, distinct from the well characterized pathway in *Escherichia coli*. The specific objectives of this work were to further characterize vitamin $B_6$ biosynthetic genes in tobacco and *Arabidopsis*, assess the regulation of $B_6$ biosynthetic genes during biotic and abiotic stress responses, and determine the antioxidant abilities of $B_6$ vitamers.

Two genes unique to the alternative pathway, *PDX1* and *PDX2*, have been described. *PDX2* has previously been shown to encode a glutaminase. Complementation studies with *E. coli* pdxA and pdxJ mutants are consistent with the hypothesis that *PDX1* is responsible for synthesis of the pyridoxine ring.

*PDX1* and *PDX2* genes were isolated and characterized from tobacco and *Arabidopsis*. Quantitative RT-PCR demonstrated that, in *Arabidopsis, PDX1* (3 copies) and *PDX2* are differentially regulated in response to high light, chilling, drought, and ozone. In tobacco, *PDX1* and *PDX2* transcript levels decreased following inoculation with the avirulent pathogen *Pseudomonas syringae* pv. *phaseolicola* and transiently increased in response to salicylic acid and methyl jasmonate. Excess vitamin $B_6$ in

tobacco leaves interfered with the development of a hypersensitive defense response and increased disease severity caused by *P. syringae* pv. *tabaci*.

$B_6$ vitamers quenched superoxide and prevented lipid peroxidation in in vitro assays.

Our findings indicate that $B_6$ vitamers have antioxidant capabilities in chemical assays and *in planta,* and that synthesis of vitamin $B_6$ is regulated during plant defense responses in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species *in vivo*.

# THE FUNCTION AND REGULATION OF $B_6$ BIOSYNTHETIC GENES BY OXIDATIVE STRESS IN PLANTS

BY

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# PERSONAL BIOGRAPHY

I was born in 1975 in Gainesville Florida, the second of two daughters for David and Nancy Denslow. After graduating from high school in 1993, I moved to Atlanta Georgia where I attended Emory University. At Emory I earned a Bachelors of Science degree with highest honors in Biology in 1997. I then moved to Chapel Hill where I spent one year learning about plants at a Hosta nursery. That year helped me realize, to my parents' relief, that school was not all that bad and in 1998 I started a dual doctoral program in Crop Science and Plant Pathology at North Carolina State University. My project, which focused on the antioxidant properties of vitamin $B_6$ and its role in the plant stress response, was completed under the direction of Dr. Margo Daub.

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# LITERATURE REVIEW

## Vitamin $B_6$

The term vitamin $B_6$ is used to collectively refer to the compound pyridoxine and its vitameric forms, pyridoxal, pyridoxamine, and their phosphorylated derivatives (Figure 1). Vitamin $B_6$ is required by all organisms. The active cofactor, pyridoxal 5'-phosphate, plays an essential role in numerous enzymatic reactions, most notably in amino acid synthesis where it serves as a cofactor for enzymes involved in decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions (Drewke and Leistner, 2001; Mittenhuber, 2001). Other significant functions include its involvement with enzymes that catalyze some steps in carbohydrate and lipid metabolism, in the synthesis of precursors to ansa antibiotics, and as a cofactor for aminocyclopropane-1-carboxylate (ACC) synthase, the regulating enzyme of ethylene biosynthesis (Drewke and Leistner, 2001; Mittenhuber, 2001). Plants, fungi, bacteria, archaebacteria, and protists synthesize pyridoxine, whereas animals must obtain it nutritionally. Vitamin $B_6$ is synthesized first via a *de novo* biosynthetic pathway that produces the basic pyridoxine molecule followed by a salvage pathway that converts between the various vitamers and their phosphorylated derivates (Drewke and Leistner, 2001; Mittenhuber, 2001). Although the enzymes for synthesis of the pyridine ring of pyridoxine are limited to organisms known to produce vitamin $B_6$, all organisms, including mammals, have the salvage pathway enzymes (Drewke and Leistner, 2001; Lam et al., 1992; Mittenhuber, 2001).

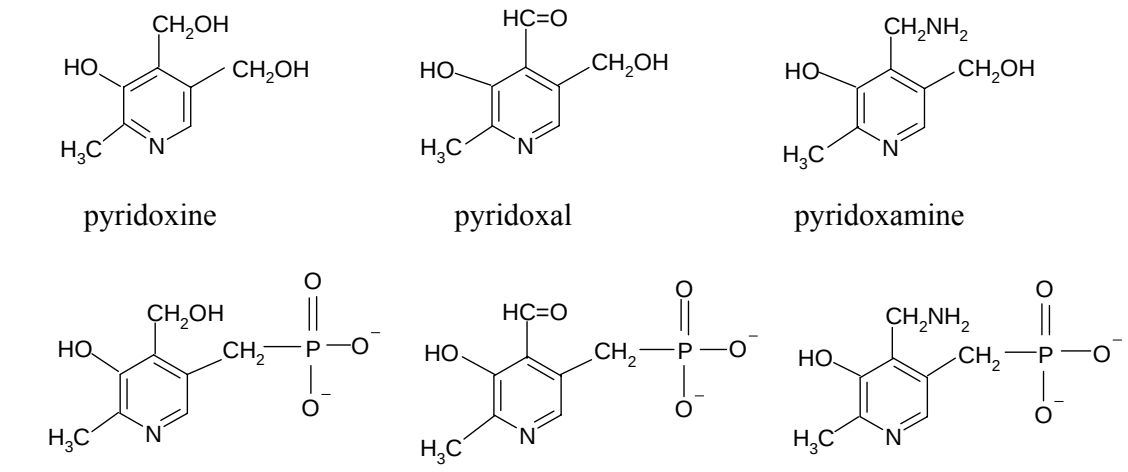


**Figure 1. Vitamers of vitamin $B_6$.** These molecules are interconverted by an efficient salvage pathway. Pyridoxal 5'-phosphate is the active cofactor.

## Vitamin $B_6$ *de novo* biosynthetic pathway

Until recently, information on *de novo* vitamin $B_6$ biosynthesis came predominantly from extensive studies of the pathway in *Escherichia coli* (Arps and Winkler, 1987; Dempsey, 1969; Drewke and Leistner, 2001; Hill and Spenser, 1986; Hill and Spenser, 1996; Hockney and Scott, 1979; Lam et al., 1992; Lam and Winkler, 1990; and Lam and Winkler, 1992; Man et al., 1996; Roa et al., 1989; Schoenlein et al., 1989; Zhao and Winkler, 1996). In this system, the $B_6$ vitamer pyridoxine 5'-phosphate is formed by the condensation and oxidation of two precursors, 4-phosphohydroxy-L-threonine, a non-proteogenic amino acid produced via enzymes common to serine biosynthesis, and 1-deoxy-D-xylulose-5-phosphate, a modified pentose produced by transketolases also involved in thiamine and isoprenoid synthesis (Hill and Spenser, 1986; Hill and Spenser, 1996; Zhao and Winkler, 1996). It is the action of the products of

two genes, *pdxA* and *pdxJ*, that combine these precursors into pyridoxine 5'-phosphate. The product of the *pdxA* gene (4-phosphohydroxythreonine dehydrogenase) mediates the oxidation and decarboxylation of 4-phosphohydroxy-L-threonine to form an unstable intermediate, 3-hydroxy-1-aminoacetone-3-phosphate (Cane et al., 1998). The ring closure between this proposed intermediate and 1-deoxy-D-xylulose-5-phosphate is catalyzed by the product of the *pdxJ* gene (pyridoxine 5'-phosphate synthase) to form pyridoxine 5' -phosphate (Drewke and Leistner, 2001; Laber et al., 1999).

While vitamin $B_6$ biosynthesis has been thoroughly characterized in *E. coli*, plants and most other $B_6$ synthesizing organisms use a different pathway for *de novo* production. This alternate $B_6$ biosynthetic pathway is a fairly new discovery and centers around *PDX1* and *PDX2*, the only unique genes identified in this pathway. Work in our laboratory in 1999 on the fungus *Cercospora nicotianae* led to the identification of these two genes, which we found were required for *de novo* biosynthesis of vitamin $B_6$ but completely unrelated in sequence to the known *E. coli* biosynthetic genes (Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001). Independently, two other groups identified *PDX1* and *PDX2* homologues in the fungus *Aspergillus nidulans* and the bacterium *Bacillus subtilis* as $B_6$ biosynthetic genes (Osmani et al., 1999; Sakai et al., 2002). Sequence database analyses demonstrated that $B_6$-producing organisms with known genomes contained either homologues to *PDX1* and *PDX2* or to *pdxA* and *pdxJ*, the *E. coli* genes (Mittenhuber, 2001; Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001). Phylogenetic analyses found that fungi, plants, archaebacteria, and some eubacteria contain homologues of *PDX1*/*PDX2*, whereas only members of the γ subdivision of the proteobacteria contain *pdxA* and *pdxJ* (Mittenhuber, 2001; Ehrenshaft et al., 1999;

Ehrenshaft and Daub, 2001). These results illustrated that the majority of $B_6$-synthesizing organisms utilize a *de novo* pathway based on *PDX1* and *PDX2* that is distinct from the pathway in *E. coli*.

Prior to the discovery of genes in the alternate pathway, there were only a few studies that suggested that the *E. coli de novo* pathway was not conserved among all vitamin $B_6$-synthesizing organisms. These studies demonstrated that yeast utilized glutamine (rather than glutamate) as the nitrogen donor (Tazuya et al., 1995) and that Ginko plants could not utilize 1-deoxy-D-xylulose as a precursor (Drewke and Leistner, 2001). Consistent with the existence of two pathways, Tanaka et al. (2000) showed that the nitrogen moiety in pyridoxine is derived from glutamine in four fungi and two prokaryotes (*Staphylococcus* and *Bacillus*), whereas it is derived from glutamate in *Pseudomonas*, *Enterobacter*, and *E. coli*. It is hypothesized that the PDX1/PDX2 pathway is the most ancient, and that ancestors to proteobacteria lost the pathway and evolved an alternative pathway utilizing *pdxA* and *pdxJ* (Mittenhuber, 2001).

As mentioned above, in *E. coli*, PDXA and PDXJ are required for the formation of the pyridine ring of pyridoxine 5'-phosphate. Specifically, PDXA is proposed to catalyze the oxidation and decarboxylation of 4-phosphohydroxy-L-threonine; PDXJ then completes the condensation and ring closure reactions (Cane et al., 1998; Drewke and Leistner, 2001). It is not known how PDX1 and PDX2 work together to produce $B_6$, or even which vitameric form of $B_6$ is first produced. Sequence analysis does not predict a function for PDX1, but does predict that it utilizes phosphorylated substrates (Galperin and Koonin, 1997; Garrido-Franco, 2003). Very recently, PDX2 has been shown to be a glutamine amidotransferase. Dong and co-workers (Dong et al., 2004) expressed the

*Saccharomyces cerevisiae* PDX2 homologue (SNO1) in an *E. coli* expression system, and directly assayed glutamine hydrolyzing activity. Independently, Bauer and coworkers (Bauer et al., 2004) determined the crystal structure of the *B. subtilis* PDX2 homologue (YaaE), and showed it to be most similar to HisH, a glutaminase involved in histidine biosynthesis. These observations confirmed the earlier prediction, based on sequence motifs, that *PDX2* encodes a glutamine amidotransferase (Galperin and Koonin, 1997), and are consistent with the identity of glutamine as the nitrogen donor (Tanaka et al., 2000).

Two independent research groups have proposed that the PDX1 and PDX2 proteins interact and form a complex. This prediction, based on two-hybrid analysis in yeast (Padilla et al., 1998) and structural similarities of the *B. subtilis* homologues to the HisH-HisF complex (Bauer et al., 2004), was recently confirmed by Dong and co-workers (Dong et al., 2004) who showed by affinity chromatography that the yeast proteins form a complex. Preliminary studies on possible precursor molecules for the PDX1/PDX2 pathway have also been done. A recent tracer labeling study in yeast documents that the C5 chain of pyridoxamine (which in *E. coli* is derived from 1-deoxy-D-xylulose-5-phosphate) is instead derived from a pentulose or pentose intermediate (Gupta et al., 2001). Studies on *in vitro* biosynthesis of vitamin $B_6$ using soluble protein fractions from spinach chloroplast stroma indicated that the spinach extracts utilize 1-deoxy-D-threo-2-pentulose as a precursor (Julliard, 1992). Taking all of these together, we hypothesize that PDX1 alone carries out the ring formation step using precursors that are different than in *E. coli* and are not yet defined. The precursors are likely phosphorylated, and may include a pentulose precursor. We further hypothesize that

PDX2 utilizes glutamine to synthesize a nitrogen-containing precursor that is a substrate for PDX1. A comparison of the PDX1/PDX2 pathway with the $B_6$ biosynthetic pathway in *E. coli* is shown in Table 1.

**Table 1. Comparison of the *de novo* vitamin $B_6$ biosynthetic pathways.**

| | **pdxA / pdxJ (*E. coli*) pathway** | ***PDX1* / *PDX2* pathway** |
|---|---|---|
| **Distribution** | γ subdivision of proteobacteria | eubacteria, archaebacteria, fungi, plants |
| **Genes / enzymes unique to pathway** | *pdxA* / 4-phosphohydroxythreonine dehydrogenase (intermediate formation)<br>*pdxJ* / pyridoxine phosphate synthase (catalyzes ring closure) | *PDX1* / unidentified, catalyzes ring closure?<br>*PDX2* / glutamine amidotransferase, synthesis of N-containing precursor? |
| **Precursors** | 4-phosphohydroxy-L-threonine<br>1-deoxy-D-xylulose-5-phosphate | N-containing intermediate<br>unidentified pentose or pentulose compound |
| **Source of Nitrogen** | glutamate | glutamine |

**Cellular localization of PDX1 and PDX2**

The cellular localization of vitamin $B_6$ synthesis in plants is not known. Our lab used GFP (green fluorescent protein) fusions to localize the PDX1 protein in *C. nicotianae* (Chung et al., 2002). Fluorescence and confocal microscopy localized the PDX1 protein to cytoplasmic vesicles in the hyphae. Western blot analysis of cell fractions using an antibody to GFP identified PDX1 in the cytoplasmic fraction,

consistent with a PSORT analysis (prediction of protein localization sites, http://psort.nibb.ac.jp), that predicts it to be a cytoplasmic protein when the analysis is run for fungal and animal proteins. Interestingly, if run in the plant protein mode, the PSORT analysis predicts the *C. nicotianae* PDX1 to localize to chloroplasts. ChloroP (www.cbs.dtu.dk/services/ChloroP/), a program that predicts the presence of chloroplast transit peptides, also predicted the *C. nicotiane* PDX1 to be localized in chloroplasts and to have an N-terminal chloroplast transit peptide. The copy of PDX1 found in *C. nicotianae* contains an N-terminal sequence that is not found in homologues in other organisms, and the plant homologues, surprisingly, do not contain known chloroplast transit peptides and thus are not predicted, based on sequence, to localize to the chloroplast. There is some evidence, however, suggesting chloroplast localization in plants. *In vitro* biosynthesis of vitamin $B_6$ was performed using soluble protein fractions from spinach chloroplast stroma (Julliard, 1992), suggesting that $B_6$ precursors are found in chloroplasts. Also, D-1-deoxy xylulose, one of the precursors for $B_6$ synthesis in *E. coli*, is found in chloroplasts as part of the isoprenoid pathway (Lichtenthaler, 1999). It is still unknown, however, if this precursor is used by PDX1 and PDX2 to synthesize $B_6$.

**Vitamin $B_6$ and oxidative stress**

While the importance of vitamin $B_6$ as a cofactor is well established, it is only in the last decade that research has linked vitamin $B_6$, often unknowingly, to oxidative stress. Gene regulation studies in numerous and diverse organisms has continually connected vitamin $B_6$ to oxidative stress, primarily through studies identifying vitamin

$B_6$ biosynthetic genes (*PDX1* and *PDX2*) or pathway recycling genes (pyridoxal kinase, oxidase, and reductases) and their products as being affected by oxidative stress conditions. In yeast (*S. cerevisiae*), *SNZ1* and *SNO1* (homologues to PDX1 and PDX2, respectively), show increased transcript and protein accumulation at entry into stationary phase, a time of high oxidative stress (Braun et al., 1996; Padilla et al., 1998). In the bacterium *Bacillus subtilis*, treatment with paraquat, an inducer of superoxide, leads to increased expression of the PDX1 homologue (YaaD) (Antelmann et al., 1997). $H_2O_2$ treatment of *Schizosaccharomyces pombe* leads to increased transcript accumulation of the *PDX2* homologue and several pyridoxal reductases (M. W. Toone, Paterson Institute for Cancer Research, Manchester, UK, personal communication). *PdxK* (pyridoxal kinase) was identified in *Arabidopsis thaliana* as *SOS4*, one of the *sos* (salt *o*verly *s*ensitive) mutants that are hypersensitive to salt stress; salt hypersensitivity in *sos4* mutants could be partially reversed by supplementing the growth medium with pyridoxine (Shi et al., 2002). *A. thaliana PdxK* was also shown to accumulate increased transcript after cold stress (Shi et al., 2002). Microarray studies recently demonstrated that the homologue to *PDX1* on chromosome 5 in *A. thaliana* is upregulated by UV-B radiation (Brosche et al., 2002). Salicylic acid and ethylene, chemical inducers of plant-pathogen defense response (a process involving high oxidative stress), boost transcript accumulation of HEVER, the *PDX1* homologue in rubber tree (*Hevea brasiliensis*) (Sivasubramaniam et al. 1995). Interestingly, the *PDX1* homologue from a marine sponge (*Suberites domuncula*) also shows responsiveness to ethylene (Krasko et al., 1999; Seack et al., 2001). In bean (*Phaseolis vulgaris*), the *PDX1* transcript was found to be upregulated by abscisic acid and after wounding (Graham et al., 2004).

More recently, in addition to the connection between oxidative stress and gene regulation, evidence has been mounting that vitamin $B_6$ is an essential antioxidant and a strong quencher of active oxygen species. In collaboration with NIEHS, our lab has shown 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 (Bilski et al., 2000). Our lab has also found that vitamin $B_6$ biosynthetic genes are necessary for resistance to singlet oxygen-generating photosensitizers in the fungus *C. nicotianae* (Ehrenshaft et al., 1998). This evidence from our lab is substantiated by findings from animal models showing the ability of $B_6$ to quench active oxygen species and to reduce or prevent the damaging effects associated with them. In blood assays, vitamin $B_6$ had three times the antioxidant activity of vitamin C and was shown to quench superoxide production (Stocker et al., 2003; Jain and Lim, 2001; Kannan and Jain, 2004). In rabbit lens cells, vitamin $B_6$ prevented protein oxidation, a cause of cataract formation (Jain et al., 2002). Vitamin $B_6$-deficient rats showed elevated inflammatory responses and lipid peroxidation (Lakshmi et al., 1991). Finally, clinical trials have shown that vitamin $B_6$ supplements prevent or delay eye and nerve damage associated with diabetes and attributed to superoxide production (Jain and Lim, 2001). It is also relevant that yeast (both *S. pombe* and *S. cerevisiae*) and *E. coli* efficiently excrete significant amounts of pyridoxine vitamers into the medium (Argoudelis , 1999; Chumnantana et al., 2001; Hirose et al., 2000; Nakano et al., 1999), an observation that suggests a function other than serving as an enzyme co-factor in amino acid synthesis.

**The plant stress response and active oxygen production**

Active oxygen species (AOS) are obligate by-products of all oxygen-utilizing organisms. Plants produce AOS during normal processes including photosynthesis, photorespiration, and metabolism, but during times of stress, excess AOS are produced, sometimes reaching damaging levels. AOS have been shown to be produced in chloroplasts, mitochondria, peroxisomes, the plasma membrane and the cell wall of plant cells (Bolwell, 1999). AOS have both beneficial and detrimental effects in plant cells. A small amount of AOS stimulates defense pathways, thus preventing damage from pathogen attack or harmful abiotic stresses. Too many AOS, however, create damaging conditions within the cell which may eventually lead to cell death (Dat et al., 2000; Mittler, 2002). Cells employ a host of compounds and enzymes to counteract the detrimental affects of active oxygen species including antioxidant enzymes such as superoxide dismutases, catalases, ascorbate peroxidase, and glutathione reductase, antioxidant molecules including reduced glutathione, ascorbate, a-tocopherol, flavonoid pigments, and carotenoids, and proteins with repair functions (heat shock proteins) (Dat et al., 2000; Davison et al., 2002; Grene , 2002; Havaux and Kloppstech, 2001; Mittler, 2002; Winkel-Shirley, 2002).

Abiotic stresses, such as high light stress, temperature stress, drought, salt, nutrient deprivation, ozone and UV irradiation all lead to increased cellular oxidative stress. Many of these stresses increase AOS in chloroplasts or mitochondria through impacting or interfering with the electron transport chains of photosynthesis and metabolism. When environmental conditions impact electron transport, such as with high

light, temperature, drought, salinity, or nutrient stress, oxygen may be energized by excess excitation energy creating singlet oxygen (in photosynthesis), or oxygen may be reduced by electrons that are backed-up from inefficient transport creating superoxide (photosynthesis and metabolism) (Fryer et al., 2002; Grene, 2002; Karpinsiki et al. 1999; Sweetlove et al., 2002) (See figure 2). Some abiotic stresses have a more directed impact on cellular oxidative stress. Ozone stress, for example, causes oxidative damage directly and is thought to primarily impact the apoplast (Grene, 2002). Ozone stress has also been shown to activate a plasma membrane localized NADPH-oxidase, creating superoxide and, through dismutase activity, hydrogen peroxide in the apoplast (Overmeyer et al., 2003). Thus, while there is overlap, various types of abiotic stresses lead to the increased production of AOS in specific regions within the cell.

The action of active oxygen in abiotic stress has been demonstrated by measurement of different active oxygen species, evidence of oxidative damage (e.g. lipid peroxidation), and increased sensitivity to stress by plants deficient in critical antioxidant enzymes (Dat et al., 2000; Fryer et al., 2002). Oxidative stress due to abiotic stresses is also demonstrated by the accumulation of antioxidant defenses (Dat et al., 2000; Davison et al., 2002; Grene , 2002; Havaux and Kloppstech, 2001; Winkel-Shirley, 2002). Perhaps due to the different regions affected by a particular stress, diverse stresses lead to induction of different defense systems, e.g. chloroplast-located Fe SODs were shown to respond to high light and UV but not to ozone, whereas Mn SODs, located in peroxisomes and the mitochondria, have been reported to respond to salt, chilling, and drought, but not high light (Grene , 2002). It has been possible to engineer plants for

increased abiotic stress resistance by transforming them to over-express various SODs or glutathione reductase (Grene, 2002).

Active oxygen also plays a central role in plant defense responses to pathogen attack (biotic stress) (Baker and Orlandi, 1995; Bolwell, 1999; Dat et al., 2000; Doke, 1983; Low and Meida, 1996; Mehdy et al., 1996). One of the earliest responses to pathogen attack is the production of superoxide, generated through an NADPH-dependent pump in the plasma membrane (Bolwell et al., 2002; Doke, 1983; De Gara et al., 2003; Keller et al., 1998; Torres et al., 1998; Torres et al., 2002). Superoxide is rapidly dismutated to hydrogen peroxide. Studies demonstrate the rapid induction of $H_2O_2$ upon exposure of plant cells and tissues to pathogens or to inducers of defense responses such as salicylic acid, jasmonic acid and ethylene (Kawano and Muto, 2000; Orozco-Cardenas and Ryan et al., 1999; Yoshioka et al., 2001; Yoshioka et al., 2003). During pathogen attack, plants also rely on the production of hydroxyl radical and nitric oxide to stimulate a defense response. AOS play a central role in plant defenses to pathogen attack acting as antimicrobial agents, as substrates in cell wall fortifications, and as signaling molecules for the activation of defense pathways (Baker and Orlandi, 1995; Bolwell, 1999; Dat et al., 2000; Doke, 1983; Low and Meida, 1996; Mehdy et al., 1996).

AOS production must be tightly controlled as over-accumulation of AOS can result in unwanted cell death, especially at sites not directly affected by pathogens. Thus plants sustain the appropriate level of AOS 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 during pathogen defenses (Mittler, 2002). There is but limited data published in this area that addresses how tissues directly affected by pathogens respond to the attack and yet protect tissues that surround the infection from undue cell death. For example, in cells directly affected by an incompatible pathogen and undergoing an HR, ascorbate peroxidase and catalase activity were suppressed (Dorey et al., 1998; Klessig et al., 2000; Mittler, 2002). Cultured tobacco cells treated with nitric oxide and hydrogen peroxide generators had decreased ascorbate peroxidase activity and decreased pools of ascorbate and glutathione pools during the HR (de Pinto et al., 2002). Further, transgenic plants that overproduce catalase showed a decreased ability to mount a defense against pathogen attack (Mittler, 2002; Polidoros et al., 2001). These studies reflect the necessity of reducing antioxidant ability in tissues directly affected by pathogens in order to mount a successful defense response.

During pathogen attack, plants also employ hormones as regulators and signaling compounds in the plant defense response. Much attention has been devoted to three of these hormones, salicylic acid, jasmonic acid, and ethylene, all of which have been heavily implicated in the pathogen stress response. The description of the involvement of these compounds in the defense response is often simplified into two antagonistic categories, with salicylic acid being involved with the response to biotrophic pathogen attack and leading to systemic acquired resistance, and ethylene and jasmonic acid both involved, though through differing mechanisms, with response to necrotrophic pathogens, insects and wound damage and leading to the induced systemic response associated with root colonization by non-pathogenic organisms (Kunkel and Brooks, 2002; Pieterse and

Van Loon, 2004). In reality, the role of these three compounds is not so easily defined, with all three showing some overlap in mode of action and pathway activation and many individualized and sometimes opposing roles. All three have been shown to stimulate AOS production (Kawano and Muto, 2000; Orozco-Cardenas and Ryan et al., 1999; Yoshioka et al., 2001; Yoshioka et al., 2003).

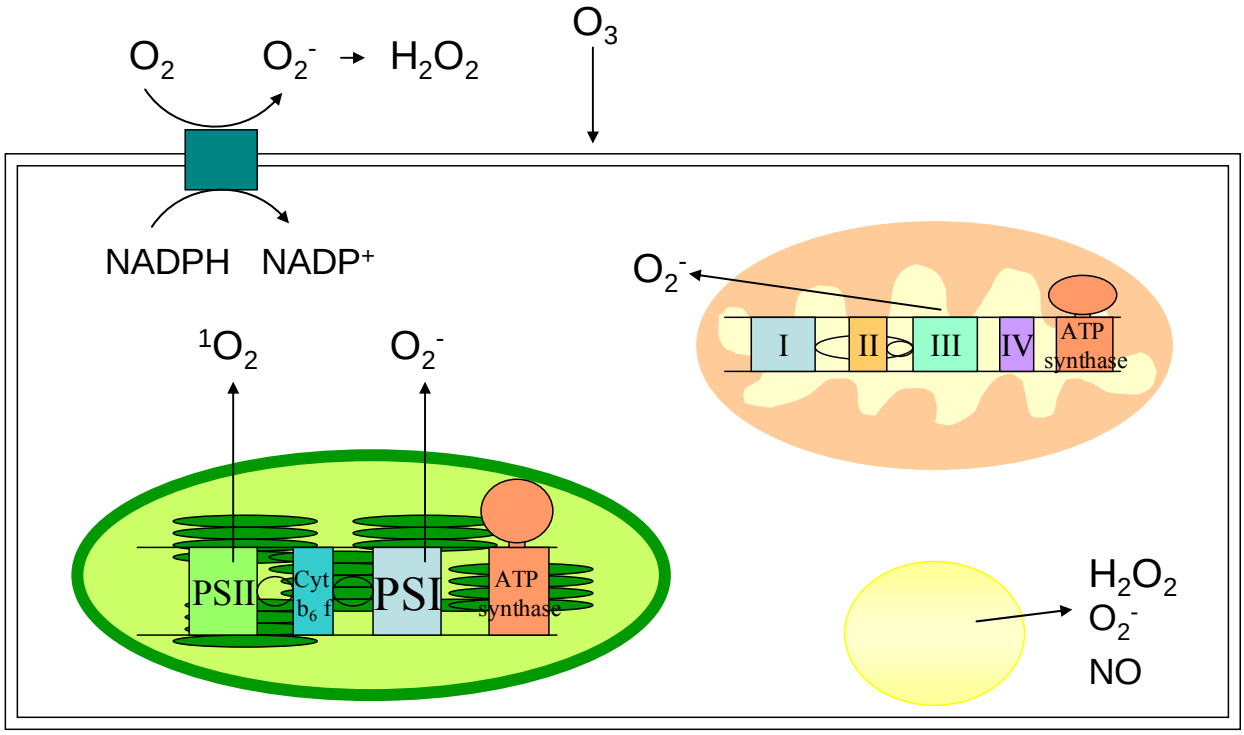


**Figure 2. Examples of sources of active oxygen species within a plant cell.** AOS are produced in the apoplast by an NADPH-dependent oxidase and peroxidases. The electron transport chains in chloroplasts and mitochondria produce AOS if energy and/or electron flow is greater than the system can process. Peroxisomes are also large producers of AOS. Green=chloroplast, pink=mitochondrion and yellow=peroxisome.

**Summary**

In the last five years, an alternate pathway for vitamin $B_6$, distinct from the thoroughly characterized pathway in *E. coli*, has been uncovered. This alternate pathway is the predominant pathway, found in the majority of $B_6$-synthesizing organisms, including plants. As this is a newly discovered pathway, little is known about $B_6$ production in plants. The last five years has also seen a growing body of data associating vitamin $B_6$ with conditions of high oxidative stress in diverse organisms, whether through regulation studies of biosynthetic genes or through studies of the $B_6$ molecules themselves. We are interested in learning more about vitamin $B_6$ biosynthesis in plants and determining if there is a link between $B_6$ production and oxidative stress in plants.

The goals of this work were 1) to determine if vitamin $B_6$ can act as an antioxidant in chemical assays and *in planta*; 2) identify and further characterize $B_6$ biosynthetic genes from tobacco and *A. thaliana*; and 3) assess their regulation during biotic and abiotic stress responses, conditions associated with the high production of AOS.

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# Chapter 1

## FUNCTIONAL COMPLEMENTATION BETWEEN THE *PDX1* VITAMIN $B_6$ BIOSYNTHETIC GENE OF *CERCOSPORA NICOTIANAE* AND *PDXJ* OF *ESCHERICHIA COLI*

# Functional complementation between the *PDX1* vitamin $B_6$ biosynthetic gene of *Cercospora nicotianae* and *pdxJ* of *Escherichia coli*

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**Abstract The pathway for de novo vitamin $B_6$ biosynthesis has been characterized in *Escherichia coli*, however plants, fungi, archaebacteria, and most bacteria utilize an alternative pathway. Two unique genes of the alternative pathway, *PDX1* and *PDX2*, have been described. *PDX2* encodes a glutaminase, however the enzymatic function of the product encoded by *PDX1* is not known. We conducted reciprocal transformation experiments to determine if there was functional homology between the *E. coli pdxA* and *pdxJ* genes and *PDX1* of *Cercospora nicotianae*. Although expression of *pdxJ* and *pdxA* in *C. nicotianae pdx1* mutants, either separately or together, failed to complement the pyridoxine mutation in this fungus, expression of *PDX1* restored pyridoxine prototrophy to the *E. coli pdxJ* mutant. Expression of *PDX1* in the *E. coli pdxA* mutant restored very limited ability to grow on medium lacking pyridoxine. We conclude that the *PDX1* gene of the alternative $B_6$ pathway encodes a protein responsible for synthesis of the pyridoxine ring.**



*Key words:* *SNZ*; *YaaD*; *SOR1*; *PYROA*; Pyridoxal-5′-phosphate; Pyridoxamine

## 1. Introduction

The term vitamin $B_6$ is used to refer collectively to the compound pyridoxine and its vitameric forms, pyridoxal, pyridoxamine, and their phosphorylated derivatives. Vitamin $B_6$ is required by all organisms and plays an essential role as a co-factor for enzymatic reactions. Plants, fungi, bacteria, archaebacteria, and protists synthesize pyridoxine. Animals and some highly specialized obligate pathogens obtain it nutritionally. All organisms have an efficient salvage pathway that interconverts between the various vitamers and their phosphorylated derivatives, but only the pyridoxine-synthesizing organisms listed above contain the de novo biosynthetic pathway [1–3].

A de novo pathway for $B_6$ synthesis was extensively characterized in *Escherichia coli* [2,4–7]. Two genes, *pdxB* and *serC*, encode enzymes for synthesis of 4-phosphohydroxy-L-threonine, one of the two precursors of the pyridoxine molecule. The second precursor is 1-deoxy-D-xylulose-5-phosphate, produced via transketolases also involved in thiamine and isoprenoid synthesis. These two precursors are joined together via the action of the products of the *pdxA* and *pdxJ* genes to form the pyridine ring of pyridoxine-5′-phosphate [2,8,9]. The product of the *pdxA* gene (4-phosphohydroxythreonine dehydrogenase) has been proposed to mediate the oxidation and decarboxylation of 4-phosphohydroxy-L-threonine to form an unstable intermediate, 3-hydroxy-1-aminoacetone-3-phosphate [10]. The *pdxJ* product (pyridoxine 5′-phosphate synthase) then catalyzes the ring closure reaction between this proposed intermediate and 1-deoxy-D-xylulose-5-phosphate to produce pyridoxine 5′-phosphate.

Work in our laboratory on the fungus *Cercospora nicotianae* identified two genes, *PDX1* and *PDX2*, that are required for de novo biosynthesis of pyridoxine but are completely unrelated in sequence to the known *E. coli* biosynthetic genes [11,12]. Independently, two other groups identified *PDX1* and *PDX2* homologues in *Aspergillus nidulans* and *Bacillus subtilis* as $B_6$ biosynthetic genes [13,14]. Sequence database and phylogenetic analyses demonstrated that fungi, plants, archaebacteria, and some eubacteria contain homologues to *PDX1* and *PDX2* and lack homologues to the *E. coli pdxA* and *pdxJ* genes, whereas only members of the γ subdivision of the proteobacteria contain *pdxA* and *pdxJ* and lack homologues to *PDX1* and *PDX2* [3,11,12]. These results demonstrated that most organisms utilize a de novo pathway for pyridoxine synthesis that is distinct from the one in *E. coli*. Consistent with the existence of two pathways, Tanaka et al. [15] showed that the nitrogen moiety in pyridoxine is derived from glutamine in four fungi and two prokaryotes (*Staphylococcus* and *Bacillus*), whereas it is derived from glutamate in *Pseudomonas*, *Enterobacter*, and *E. coli*.

Very recently, the enzymatic function of the product of *PDX2* has been demonstrated. Dong and co-workers [16] expressed the *Saccharomyces cerevisiae PDX2* homologue (*SNO1*) in an *E. coli* expression system, and directly assayed glutamine hydrolyzing activity. Independently, Bauer and co-workers [17] determined the crystal structure of the *B. subtilis* PDX2 homologue (YaaE), and showed it to be most similar to HisH, a glutaminase involved in histidine biosynthesis. These observations confirmed the earlier prediction, based on sequence motifs, that *PDX2* encodes a glutamine amidotransferase [18], and are consistent with the identity of glutamine as the nitrogen donor [15].

In contrast to *PDX2*, sequence analysis of the predicted PDX1 protein provides no clues to its function. Two indepen-

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dent research groups have proposed, based on two-hybrid analysis in yeast [19] and structural similarities of the *B. subtilis* homologues to the HisH–HisF complex [17], that the PDX1 and PDX2 proteins interact and form a complex. This prediction was recently confirmed by Dong and co-workers [16] who showed by affinity chromatography that the yeast proteins form a complex. They also proposed that the complex functions as a glutamine amidotransferase, with PDX2 acting as the glutaminase. However, *PDX1* and *PDX2* are the only unique genes identified in the alternative pathway, arguing that *PDX1* has an additional function.

The purpose of this research was to use cross-complementation analysis to determine if there is functional homology between the products encoded by the *C. nicotianae PDX1* gene and the *E. coli pdxA* and *pdxJ* genes, which would provide clues to the function of PDX1. Our research indicates a functional relationship between *PDX1* and *pdxJ*, suggesting that PDX1 is involved in the pyridoxine ring closure reaction.

## 2. Materials and methods

### 2.1. Fungal strains, cultural conditions and transformation

Wild type *C. nicotianae* strain ATCC #18366 and the *pdx1* mutant strain CS8 [20,21] were maintained on malt medium [22] at 28°C in the dark. The vectors pHYGPRO and pBarGPE1 [23] were used to transform *C. nicotianae* for expression of *pdxA* and *pdxJ* (see vector construction below). Mycelial protoplasts from *C. nicotianae* were prepared and transformed as previously described [24,25]. Transformants were selected on medium containing 200 μg/ml hygromycin (pHYGPRO) and/or 50 μg/ml bialaphos (pBarGPEI). Experiments to determine pyridoxine auxotrophy used a minimal medium [22] containing bacteriological agar (Sigma, St. Louis, MO, USA) with and without the addition of 1 μg/ml pyridoxine. Growth was determined by transferring fungal mycelium as a toothpick point inoculation and measuring the increase in colony diameter after 4 days at 28°C.

### 2.2. E. coli strains, culture conditions, and transformation

*E. coli* strains and clones were provided by Malcolm E. Winkler, University of Indiana, Bloomington, IN, USA. Strains NU812 and TX1918 are mutant, respectively, in *pdxA* [4] and *pdxJ* [7], and are derived, respectively, from parent strains NU426 and NU816. Both mutants carry a kanamycin resistance marker. The *pdxA* and *pdxJ* genes were provided on plasmids pNU244 and pNU199, respectively, both containing an ampicillin resistance marker. Standard methods [26] were used for transformation of *E. coli* with vector pBluescript II KS$^+$ for expression of *PDX1* (see vector construction below). Transformants were initially screened on minimal Vogel–Bonner 1XE medium (E medium) containing 0.01 mM $FeSO_4$ [27] with and without 1 μg/ml pyridoxine. To quantify growth, strains were grown in 5 ml of liquid E medium+1 μg/ml pyridoxine overnight at 37°C with shaking at 200 rpm. One ml of culture was centrifuged at 5220 RCF for 5 min and the pellet resuspended in 2 ml deionized $H_2O$. The density of the suspension was adjusted to an OD of 0.6 at 600 nm. Ten and 100 μl of each inoculum suspension was plated onto plates of solid E medium with and without 1 μg/ml pyridoxine. Cultures were incubated for 24 and 48 h at 37°C. Bacterial lawns were washed from the plates with 2 ml of deionized $H_2O$, diluted 1:20, and the $OD_{600}$ of the diluted suspension determined.

### 2.3. Gene cloning

The *C. nicotianae PDX1* gene was amplified from cosmid clone 18E1 [25] using *Taq* DNA polymerase (Promega, Madison, WI, USA) with an annealing temperature of 60°C for 30 cycles. *PDX1* was amplified utilizing primers 5′-ATGGCCTGTAACGGAACTTC-3′ and 5′-TGGCTGGTAGATGCTGCAAA-3′. The amplified gene was ligated into the Sma site of the *E. coli* vector pBluescript II KS$^+$ (Stratagene, La Jolla, CA, USA) behind the β-galactosidase promoter according to the manufacturer's recommendations. The resulting construct was sequenced and used for transformation of *E. coli* mutants NU812 and TX1918. The transformants were selected on media containing ampicillin and kanamycin.

For cloning of *pdxA* and *pdxJ* into fungal vectors, primers were designed using sequence data in the National Center for Biotechnology Information (NCBI) database. The *pdxA* gene was amplified from strain NU1350 containing the pNU244 plasmid using primers 5′-GCGCCTACGTTAAAATCCTGA-3′ and 5′-CTAAGTGGCCCTG-GTGGACT-3′. The amplified gene was ligated into the Sma site of fungal vector pBarGPE1 behind the constitutive *A. nidulans gpdA* (glyceraldehyde-3-phosphate) promoter [28]. This vector contains the complete phosphinothricin acetyltransferase *bar* gene [29] used as a selectable marker behind the *trpC* promoter, conferring resistance to bialaphos. The *pdxJ* gene was amplified from strain NU1062 containing plasmid pNU199 using primers 5′-CCTAAGCGAACGG-TGAAAAC-3′ and 5′-TCCACAATATCCGTGCCTAA-3′. The fungal transformation vector, pHYGPRO, was constructed by utilizing pGEM-3Zf(+) (Promega) and inserting a fragment containing the hygromycin B phosphotransferase (*hph*) gene from *E. coli* [30] into the Nde site, allowing for selection with hygromycin. The amplified *pdxJ* was cloned into the vector behind Promoter 1 from pBar3 [29]. The pHYGPRO+pdxJ and pBarGPE1+pdxA constructs were sequenced, and used for transformation of the *C. nicotianae pdx1* (CS8) mutant. Transformants were selected and screened as described above. All plasmids were manipulated in *E. coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) and placed in LB medium as recommended. Standard methods were used for endonuclease digestion, ligation, construction of plasmids, and *E. coli* transformation [26].

### 2.4. Transgene expression

Transgene expression was determined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from lyophilized fungal tissue using Tri-Reagent (Sigma) following the manufacturer's protocol and adding a second phenol–chloroform extraction. RNA was resuspended in RNAsecure (Ambion, Austin, TX, USA) and DNase-treated twice with DNA-*free* (Ambion) for 1 h. RT-PCR was performed in a ratio of 1 ng RNA per μl reaction mix using the Access RT-PCR System (Promega). Gene-specific primers (0.2 pM/μl final concentration) used (from IDT) were: *pdxA* 5′-CGG-TCGAACTGGTTGTTTG-3′ and 5′-CACTGATGTGCGAATAAA-GG-3′; *pdxJ* 5′-TGAAGATCGCCGTCACATTAC-3′ and 5′-GTC-ATCACTGCACGACCAATA-3′. Thermocycler parameters for the reverse transcription reaction were 48°C for 45 min, 94°C for 2 min, followed by 30 cycles of PCR at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min.

## 3. Results and discussion

*E. coli pdxA* and *pdxJ* mutants were transformed with the *C. nicotianae PDX1* gene, and the resulting transformants were tested for pyridoxine auxotrophy. Plates of E medium with or without pyridoxine were inoculated with 10 or 100 μl of inoculum, and the resulting growth assayed spectrophotometrically at 24 and 48 h (Fig. 1). All strains (wild type, mutants, transformants) grew on E medium+pyridoxine and reached similar levels of growth within 24 h (Fig. 1, top). On medium lacking pyridoxine, wild type strains grew within 24 h, whereas the *pdxA* and *pdxJ* mutants failed to grow at either inoculum concentration up to 48 h (Fig. 1, bottom). *PDX1* clearly restored pyridoxine prototrophy to the *pdxJ* mutant, although growth was slower than that of the wild type strain. By contrast, growth of the *pdxA* mutant transformed with *PDX1* was very poor and never reached wild type levels even within 48 h. The differential ability of *PDX1* to complement the *pdxA* and *pdxJ* mutants argues against the possibility that expression of PDX1 simply by-passes the normal *E. coli* pathway through catalyzing a reaction using other substrates present in *E. coli*, and supports the hypothesis that PDX1 and pdxJ have functional homology.

Our complementation results are consistent with a hypothesis that the PDX1 protein utilizes the normal PdxJ precur-

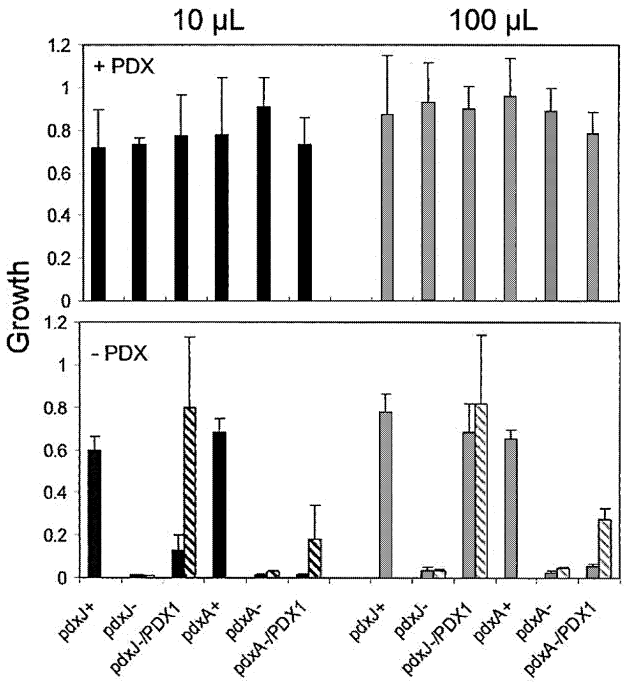


Fig. 1. Growth of *E. coli pdx* mutants transformed with the *C. nicotianae PDX1* gene. Strains were inoculated onto minimal E medium plates with 1 µg/ml pyridoxine (+PDX) or E medium alone (−PDX). Cultures started with 10 µl (black bars, left) or 100 µl (gray bars, right) of inoculum and incubated for 24 (solid bars) or 48 (hatched bars) h. Growth was assayed as described in Section 2. Strains tested were the wild type parent of the *pdxJ* mutant (pdxJ+), the *pdxJ* mutant (pdxJ−), the *pdxJ* mutant transformed with *PDX1* (pdxJ−/PDX1), the wild type parent of the *pdxA* mutant (pdxA+), the *pdxA* mutant (pdxA−), and the *pdxA* mutant transformed with *PDX1* (pdxA−/PDX1). Growth of wild type strains or of cultures grown on E medium+pyridoxine were assayed only at 24 h. Data shown are the means of two independent experiments. Error bars represent S.E.M.

sors, but has a lower affinity for them. All evidence to date supports the conclusion that the precursor substrates specific for the different biosynthetic pathways utilized by PDX1 and PdxJ are different. Tracer labeling studies in two yeasts, *S. cerevisiae* and *Candida utilis*, support a pentose or pentulose precursor for the $C_5$ unit and an intact triose for the $C_3$ unit [31,32] rather than the 1-deoxy-D-xylulose-5-phosphate and 4-phosphohydroxy-L-threonine precursors in *E. coli*. Consistent with the above, *serC*, required for production of 4-phosphohydroxy-L-threonine in *E. coli* is not required for $B_6$ synthesis in *B. subtilis* [33].

Interestingly, recent structural studies of the PdxJ protein have demonstrated significant similarities to earlier predictions about the PDX1 family of proteins. Garrido-Franco and co-workers [34] identified the PdxJ protein as a β/α-barrel protein that contains two different phosphate binding sites. Earlier, Galperin and Koonin [18] predicted, based on sequence motifs, that the PDX1 group of proteins were β/α-barrel proteins with a single phosphate binding site.

Reciprocal experiments were also conducted to express the *E. coli pdxA* and *pdxJ* genes in the *C. nicotianae pdx1* mutant. Surprisingly, none of the transformants were restored to pyridoxine prototrophy, either by transformation with *pdxJ* alone, or in combination with *pdxA* (Fig. 2). RT-PCR experiments showed the expected 0.5- and 0.8-kb bands for *pdxA*

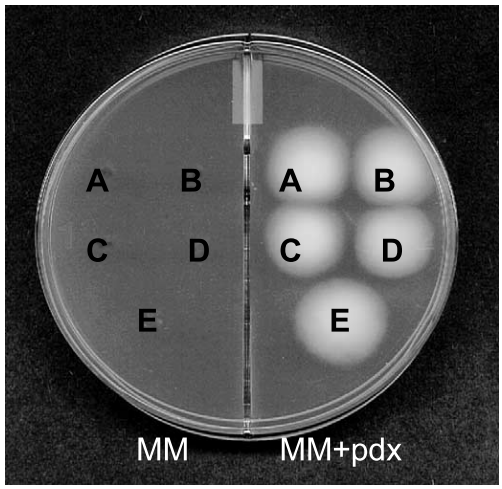


Fig. 2. *C. nicotianae pdx1* mutant transformed with *E. coli pdx* genes grown on minimal medium (MM, left) and minimal medium plus 1 µg/ml pyridoxine (MM+pdx, right). Strains transformed with: (A) *pdxA*, (B) *pdxA* vector control, (C) *pdxJ*, (D) *pdxJ* vector control, and (E) double transformant with *pdxA* and *pdxJ*.

and *pdxJ*, respectively, confirming expression of the transgenes in the transformants (Fig. 3). Thus lack of complementation was not due to lack of expression of the transgenes.

Thus, although *PDX1* complements the *pdxJ* mutation, *pdxJ*, either alone or in combination with *pdxA*, cannot complement the *pdx1* mutation. This lack of complementation may be due to the inability of the *E. coli* proteins to utilize the substrates of PDX1 and the lack of the normal *E. coli* substrates in *C. nicotianae*. There is no evidence at this time that fungi produce the normal substrates for the *E. coli* PdxA and PdxJ proteins. The $C_5$ intermediate, 1-deoxy-D-xylulose 5-phosphate, is a component of the non-mevalonate pathway for isoprenoid synthesis that operates in bacteria, green algae and plant chloroplasts [35], but we have been unable to find a report of its presence in fungi, or of 4-phosphohydroxy-L-threonine. Thus it is likely that *C. nicotianae* lacks the precursors necessary for functioning of PdxA and PdxJ.

In summary, we have shown that the *PDX1* gene of *C. nicotianae* complements the *E. coli pdxJ* mutation. These results support the conclusion that the PDX1 protein is involved in the formation of the pyridoxine ring.

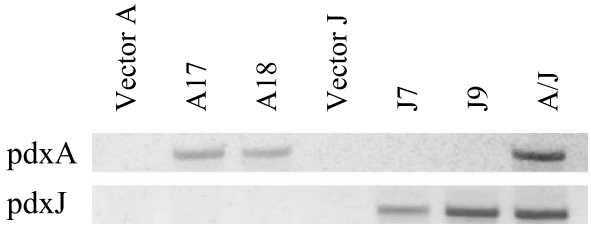


Fig. 3. Expression of the *E. coli pdxA* and *pdxJ* transgenes in *C. nicotianae pdx1* mutant. RNA was extracted from the fungal tissue and amplified by RT-PCR. Strains tested are: vector A = mutant transformed with plasmid pBarGPE1; A17, A18 = two independent transformants with pBarGPE1+pdxA; vector J = mutant transformed with plasmid pHYGPRO; J7, J9 = two independent transformants with pHYGPRO+pdxJ; A/J = mutant transformed with both pBarGPE1+pdxA and pHYGPRO+pdxJ.

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# Chapter 2

## REGULATION OF BIOSYNTHETIC GENES AND ANTIOXIDANT PROPERTIES OF VITAMIN $B_6$ VITAMERS DURING PLANT DEFENSE RESPONSES

**REGULATION OF BIOSYNTHETIC GENES AND ANTIOXIDANT PROPERTIES OF VITAMIN $B_6$ VITAMERS DURING PLANT DEFENSE RESPONSES**


## Abstract

Vitamin $B_6$ plays an essential role as a cofactor in enzymatic reactions, but has only recently been linked to cellular oxidative stress. We investigated the possible role of this vitamin in oxidative responses linked to plant defense. $B_6$ vitamers (pyridoxine, pyridoxal, and pyridoxamine) effectively quenched superoxide and had antioxidant activity when assayed *in vitro*. The *de novo* $B_6$ biosynthetic genes (*PDX1* and *PDX2*) were identified in *Nicotiana tabacum* cv. 'Burley 21' and their transcript abundance was assayed during defense responses. In tobacco leaves exposed to the avirulent pathogen *Pseudomonas syringae* pv. *phaseolicola*, transcript abundance varied depending on the tissue assayed. In tissue undergoing a hypersensitive response (HR), *PDX1* and *PDX2* transcript abundance decreased, but in the tissue surrounding the HR, *PDX1* transcript abundance showed a small, transient increase and *PDX2* transcript abundance remained near control levels. Treatment of tobacco with salicylic acid and methyl jasmonate, two chemical inducers of plant defense responses, resulted in a transient increase in *PDX1* transcript abundance for both chemical treatments, and an increase in *PDX2* transcript abundance for the methyl jasmonate treatment only. Infiltration of leaves with pyridoxine mixed with inoculum of *P. syringae* pv. *phaseolicola* or with the virulent pathogen *P. syringae* pv. *tabaci*, resulted in a delayed HR response and increased disease symptoms, respectively. Our findings indicate that synthesis of vitamin $B_6$ is regulated during plant defense

responses in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species *in vivo*.

## Introduction

Vitamin $B_6$, 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 (Drewke and Leistner, 2001; Mittenhuber, 2001). Other significant functions include its involvement in carbohydrate and lipid metabolism, in producing some antibiotic precursors, and in synthesizing aminocyclopropane-1-carboxylate (ACC) (Drewke and Leistner, 2001; Mittenhuber, 2001).

While the importance of vitamin $B_6$ as a cofactor is well established, it is only in the last decade that research has linked vitamin $B_6$, often unknowingly, to oxidative stress. The earliest suggestions of a connection between vitamin $B_6$ and cellular antioxidant defense were based on regulation studies on homologues of the two genes now known to be responsible for the *de novo* biosynthesis of vitamin $B_6$ in most organisms, *PDX1* and *PDX2*. Almost every published study involving *PDX1* and *PDX2* homologues related directly or indirectly to their regulation during oxidative stress. In yeast (*Saccharomyces cerevisiae*), the *PDX1* and *PDX2* homologues, *SNZ1* and *SNO1*, show increased transcript and protein accumulation at entry into stationary phase, a time of high oxidative stress (Braun et al., 1996; Padilla et al., 1998). In the bacterium *Bacillus*

*subtilis*, there is increased protein accumulation of the PDX1 homologue upon treatment with paraquat, an inducer of superoxide (Antelmann et al., 1997). $H_2O_2$ treatment of *Schizosaccharomyces pombe* leads to increased transcript accumulation of the *PDX2* homologue and several pyridoxal reductases, genes whose products are a part of the vitamin $B_6$ salvage pathway (M. W. Toone, Paterson Institute for Cancer Research, Manchester, UK, personal communication), the pathway that cycles between the different vitamers. Pyridoxal kinase, another component of the vitamin $B_6$ salvage pathway, has been connected to salt tolerance and cold responses in *Arabidopsis thaliana* (Shi et al., 2002), and one of the *Arabidopsis PDX1* homologues (on chromosome 5) shows increased transcript accumulation after exposure to UV-B radiation (Brosche et al., 2002).

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 (Bilski et al., 2000). Further, vitamin $B_6$ biosynthetic genes are necessary for resistance to singlet oxygen-generating photosensitizers in the fungus *Cercospora nicotianae* (Ehrenshaft et al., 1998). 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 (Stocker et al., 2003) and was shown to quench superoxide production (Jain and Lim, 2001; Kannan and Jain, 2004). Vitamin $B_6$ prevented protein oxidation in rabbit lens cells (a cause of cataract formation) (Jain et al., 2002), and prevented the elevated inflammatory responses and lipid peroxidation characteristic of vitamin $B_6$-deficient rats (Lakshmi et al., 1991). Clinical trials have shown that vitamin $B_6$ supplements prevent or

delay eye and nerve damage associated with diabetes and attributed to superoxide production (Jain and Lim, 2001).

We are interested in the possible antioxidant activity of vitamin $B_6$ during plant pathogen 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 (Baker and Orlandi, 1995; Bolwell, 1999; Dat et al., 2000; Doke, 1983; Lamb and Dixon, 1997; Low and Meida, 1996; Mehdy et al., 1996). But 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 (Mittler, 2002). There is limited data published that addresses how tissues directly affected by pathogens respond to the attack while still protecting tissues that surround the infection from undue cell death.

The goal of this work was to determine if vitamin $B_6$ has a role in cellular antioxidant protection during times of high AOS production during the pathogen defense response. 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* (Sivasubramaniam et al., 1995). No further studies on vitamin $B_6$ and plant defense responses have been published,

however. Here we report on the ability of vitamin $B_6$ vitamers to quench superoxide and prevent lipid peroxidation, the regulation of $B_6$-synthesizing genes following pathogen inoculation and salicylic acid and methyl jasmonate treatment, 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*.

## Results

### *Vitamin $B_6$ quenches superoxide and has antioxidant activity*

Previous studies addressing the ability of $B_6$ 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 (Figure 1). Pyridoxal showed strong quenching activity, with 1 mM pyridoxal having equivalent quenching ability as 1 unit superoxide dismutase (SOD). Pyridoxine and pyridoxamine also quenched superoxide, but to a lesser extent.

The antioxidant activity of $B_6$ vitamers was tested by assessing their ability to prevent lipid peroxidation in an assay measuring the coupled oxidation of β-carotene and linoleic acid (Figure 2). All three vitamers showed significant antioxidant activity 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 $B_6$ vitamers are magnified by the experimental system; BHT is more soluble in the liposomes than the water-soluble $B_6$ vitamers, increasing its effectiveness.

*Isolation of PDX1/PDX2 genes from tobacco*

In order to assess possible regulation of *de novo* vitamin $B_6$ biosynthetic genes during defense responses, homologues to *PDX1* and *PDX2* were identified in Burley 21 tobacco. Southern analysis suggested the presence of one copy of both *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 the Methods section, 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). As is common for all homologues identified to date, the tobacco *PDX1* homologues are composed of one exon. 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. *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**.**

*Regulation of tobacco vitamin $B_6$ biosynthetic genes during pathogen response*

Using northern analysis (*PDX1* only) and quantitative RT-PCR (*PDX1* and *PDX2*), we assessed *PDX1* and *PDX2* transcript abundance in tobacco during the defense response to *Pseudomonas syringae* pv. *phaseolicola*, a pathogen that is virulent on bean, but avirulent on tobacco due to the induction in tobacco of the hypersensitive defense response (HR). We chose to use this pathogen as *Pseudomonas* species contain an alternate vitamin $B_6$ biosynthetic pathway similar to *E. coli* and thus do not contain *PDX1* or *PDX2* homologues, ensuring that we were only measuring plant transcript. The two versions of *PDX1* were measured in total as we did not differentiate between the versions through northern analysis or qPCR. Tobacco leaves were vacuum-infiltrated or syringe-infiltrated with $10^8$ cfu/mL of the bacterium. The infiltrated regions showed chlorosis and bronzing at 24 hours and necrosis by 48 hours, symptoms indicative of the HR (Figure 3). Transcript accumulation was assessed in the infiltrated tissue and in the 5 mm region surrounding the infiltrated tissue.

Through northern analysis, an increase in *PDX1* transcript abundance was observed within the infiltrated area from 2-4 hours post-infiltration, but then decreased (Figure 4). An increase in *PDX1* transcript was also observed in the water control, though less pronounced and for a longer time frame. 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 general

hypoxia stress caused by water infiltration or to wounding. qPCR confirmed the northern results (Figure 5a). *PDX1* transcript levels were almost identical for the tissue infiltrated with water and the avirulent pathogen during early stages of the defense response, with pathogen-infiltrated tissue showing an average 1.16-fold increase in transcript abundance as compared to water-infiltrated tissue 2 hours post-infiltration. By 6 and 10 hours post-infiltration, however, transcript abundance of *PDX1* dropped to half those of the water controls.

*PDX2* proved difficult to assess by northern analysis due to its low expression level so expression levels were only measured using qPCR. Unlike *PDX1* transcript levels, which mimicked those seen in water control two hours post-infiltration, *PDX2* transcript abundance started to drop soon after infiltration, falling to 0.8-fold decrease from water infiltrated tissue by two hours post-infiltration (Figure 5b). Levels of *PDX2* transcript at 6-10 hours post-infiltration were comparable to the decrease observed for *PDX1*.

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*) 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 hours post-infiltration (Figure 5c). These results show that in tissue undergoing an HR, the decrease in transcript abundance of $B_6$ biosynthetic genes is not simply due to the cells' inability to transcribe genes during an HR.

For the tissues in a 5 mm region bordering the infiltrated region, we saw a more variable response by qPCR. When averaged over four experiments, the trend in transcript abundance shows that *PDX1* 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 hours post-infiltration respectively (Figure 6a).

Variability was highest at the 24 hour time point. *PDX2* transcript levels also stayed near water-control values (Figure 6b). *PAL* transcript abundance, by contrast, was increased significantly in the 5 mm border region, reaching maximum levels (17.5-fold increase over water control) at 10 hours post-infiltration (Figure 6c).

*Regulation of tobacco vitamin $B_6$ biosynthetic genes during salicylic acid and methyl jasmonate treatment*

*PDX1* and *PDX2* transcript abundance was also determined using northern analysis (*PDX1* only) and qPCR (*PDX1* and *PDX2*) following treatment with salicylic acid and methyl jasmonate, chemical inducers of plant defense responses (Figures 7 and 8). Leaf tissue of tobacco plants sprayed with 2 mM or 5 mM salicylic acid showed a dose-dependent increase in *PDX1* transcript abundance 6 hours after treatment, followed by a decline at 24 and 48 hrs (Figure 7). As a control, *PR-1a* transcript levels began increasing at 6 hrs and continued to increase through 48 hours. Results for *PDX1* were confirmed by qPCR; *PDX1* transcript reached an average of 3-fold (2 mM) and 4.3-fold (5 mM) increase over the water control at 6 hours, and then declined, reaching control expression levels or lower, by 24 hours (Figure 8a). *PDX2* transcript abundance, by contrast, remained at or below control levels for all time points (Figure 8b). Tobacco plants sprayed with 1 mM methyl jasmonate showed increased *PDX1* and *PDX2* transcript abundance at 24 hours, dropping back to control levels by 48 hours (Figure 8c).

*Vitamin $B_6$ interferes with the plant-pathogen defense response*

As vitamin $B_6$ biosynthesis is both up- and down-regulated during plant defense responses, we wished to test the effect of modified levels of vitamin $B_6$ on a plant's response to both virulent and avirulent pathogens. We initially attempted to increase the levels of vitamin $B_6$ in tobacco by constitutive expression of fungal homologues of *PDX1* and *PDX2*, however these efforts were unsuccessful (Herrero and Daub, unpublished). We have also been unsuccessful at finding knock-out lines in *Arabidopsis* with altered $B_6$ levels as homozygous knock-outs for *PDX2* are lethal and heterozygous knock-outs do not have altered $B_6$ content when grown on soil (Rueschhoff and Daub unpublished). We thus opted to temporarily increase pyridoxine levels in leaf tissue through co-infiltration along with the *Pseudomonas* inoculum. Possible toxicity of pyridoxine toward the bacterial pathogens *P. syringae* pv. *phaseolicola* (the avirulent strain) as well as the virulent pathovar *P. syringae* pv. *tabaci* was first tested; 100 mM pyridoxine had no effect on survival of either strain *in vitro* (data not shown). Tobacco plants were then co-infiltrated with pyridoxine mixed with bacterial inoculum.

Pyridoxine treatment led to a delayed hypersensitive defense response in the avirulent interaction (Figure 9a). In the treatment with bacteria alone, symptoms of the HR (tissue collapse followed by necrosis) were first visible at 10 hours. In the treatment with pyridoxine, there were no symptoms at 10 hours, and only limited tissue wilting at 24 hours. Some necrosis developed by 72 hours, but was less extensive than in the treatment without pyridoxine. Surprisingly, *in vivo* bacterial growth curves showed that the delay in the defense symptoms was not correlated with populations of bacteria in the tissues; in both treatments, bacterial populations *in planta* start high and then decrease at 8-12 hours post-infiltration. Although there was a slight delay in

the timing of the population drop in the water control (10 hours for the water control versus 8 hours for pyridoxine treatment), both pyridoxine and non-pyridoxine treated leaves show a decrease in bacterial numbers to similar levels (Figure 9b). These results suggest that the delay in the HR is not due to an effect on bacterial numbers.

Tobacco plants infiltrated with the virulent bacterium *P. syringae* pv. *tabaci* plus pyridoxine showed increased disease symptoms compared to those infiltrated with bacteria alone (Figure 10a). At 72 hours, the pyridoxine-treated plants had stronger chlorosis with more areas of necrosis. *In vivo* bacterial growth curves, however, showed no correlation with symptom expression. Bacteria in the pyridoxine treatment, although resulting in more severe symptoms, accumulated to lower population levels than in the water control (Figure 10b). Thus, as with the avirulent interaction, the effect of pyridoxine on disease symptoms was not due to an effect on bacterial numbers.

The delayed HR and enhanced symptom expression in the virulent interaction were most pronounced when using 100 mM pyridoxine. In order to determine if this treatement level resulted in artificially high concentrations of pyridoxine in leaf tissue, we used a bioassay to measure the amount of total vitamin $B_6$ in the infiltrated tissues. Total $B_6$ content was determined by extracting tissue, converting the vitamers to pyridoxine by acid hydrolysis and phosphatase treatment, and 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 $B_6$ content. Leaves were infiltrated and assayed immediately upon disappearance of the water-soaking symptoms (2 hours). Pyridoxine infiltrated tissue contained an average 185 nmoles pyridoxine per gram leaf tissue as compared to 153 nmoles pyridoxine per gram leaf tissue for non-infiltrated tissue and 130 nmoles pyridoxine per gram leaf tissue for

the water-infiltrated control. Thus pyridoxine treatment significantly increased the concentration of pyridoxine in the leaf tissue, but still within physiologically acceptable levels.

## Discussion

Previous studies in animal systems have connected pyridoxine with protection against oxidative damage (Lakshmi et al., 1991; Jain et al., 2002; Jain and Lim, 2001; Kannan and Jain, 2004; Stocker et al., 2003). We confirmed these studies and tested the $B_6$ vitamers pyridoxine, pyridoxal and pyridoxamine with controlled *in vitro* studies. Our lab has previously shown that $B_6$ vitamers are potent quenchers of singlet oxygen (Bilski et al., 2000). The work described here confirms that $B_6$ 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, generated through an NADPH-dependent pump in the cell membrane (Bolwell et al., 2002; Doke, 1983; De Gara et al., 2003; Keller et al., 1998; Torres et al., 1998; Torres et al., 2002). During a defense response, both to virulent and avirulent 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 (Baker and Orlandi, 1995; Bolwell, 1999; Dat et al., 2000; Doke, 1983; Lamb and Dixon, 1997; Low and Meida, 1996; Mehdy et al., 1996). When pyridoxine levels were increased in leaf tissue by infiltration, we observed a delay

in the hypersensitive response during exposure to an avirulent pathogen and a pronounced increase in disease symptoms during exposure to a virulent pathogen. We hypothesize that defense related changes, caused by increased production of AOS, were hindered by pyridoxine, supporting a hypothesis that $B_6$ vitamers can act as antioxidants in plants and may act as important modulators of redox status during pathogen defense response.

Congruent with pyridoxine interfering with a pathogen defense response, our regulation studies show that *de novo* vitamin $B_6$ 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 salicylic acid, methyl jasmonate, or an avirulent pathogen leading to an HR, we saw an initial increase followed by a return to control levels or, in some cases, a sharp decrease in transcript accumulation for these proteins, 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 during an HR, as we saw large increases in transcript for *PAL*, a gene shown to accumulate in HR tissue, whose product is the key regulator of the secondary metabolism pathways leading to the production of many defense compounds (Dorey et al., 1997). 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 $B_6$ biosynthetic genes in tissue undergoing an HR is not simply due to the cells' inability to transcribe genes during an HR, but is presumably due to an active process to decrease $B_6$ 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 hours post-infiltration, suggestive of a need for vitamin $B_6$ in the surrounding tissue, perhaps involved in protection from AOS damage.

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 showing altered activities during plant defense. For example, ascorbate peroxidase and catalase activity were suppressed in cells directly affected by an avirulent pathogen and undergoing an HR (Dorey et al., 1998; Klessig et al., 2000; Mittler, 2002). Cultured tobacco cells treated with NO and $H_2O_2$ generators had decreased ascorbate peroxidase activity and decreased pools of ascorbate and glutathione pools during the HR (de Pinto et al., 2002). Further, transgenic plants that overproduce catalase showed a decreased ability to mount a defense against pathogen attack (Mittler, 2002; Polidoros et al., 2001). These studies reflect the necessity of reducing antioxidant ability in order to mount a successful defense response.

In all of our infiltration experiments, including water controls, we observed an increase in transcript accumulation for *PDX1* approximately 2-6 hours post-treatment. We hypothesize that this is a response to water stress or wounding. A recent study showed that a *PDX1* homologue from *P. vulgaris* showed increased transcript abundance in response to wounding (Graham et al., 2004). Recent data from our lab on gene regulation in *Arabidopsis* suggest that $B_6$ *de novo* biosynthetic genes are also highly regulated by light cycles and abiotic stress conditions. These results support a role for vitamin $B_6$ in protection against other AOS-producing scenarios.

The $B_6$ pathway is poorly characterized in plants. While vitamin $B_6$ biosynthesis has been thoroughly characterized in *E. coli (*Arps and Winkler, 1987; Dempsey, 1969; Drewke and Leistner, 2001; Hill and Spenser, 1986; Hill and Spenser, 1996; Hockney and Scott, 1979; Lam et al., 1992; Lam and Winkler, 1990; and Lam and Winkler, 1992; Man et al., 1996; Roa et al., 1989; Schoenlein et al., 1989; Zhao and Winkler, 1996), plants and most other $B_6$ synthesizing organisms use a different pathway for *de novo* production. We identified the two key genes in

this pathway in Burley 21 tobacco, *PDX1* and *PDX2*. The *PDX2* gene product has been shown to be a glutaminase (Bauer et al., 2004; Dong et al., 2004), and is hypothesized to be involved in production of the nitrogen-containing substrate for PDX1. It is known that PDX2 and PDX1 proteins form a complex (Dong et al., 2004), but the PDX1 sequence provides no clues to its function. We recently demonstrated that *PDX1* complements a *pdxJ* mutation in *E. coli*, strongly suggesting that PDX1 catalyzes the ring closure reaction of the pyridoxine molecule (Wetzel et al., 2004). In 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* ecotype Columbia has three full copies and one partial copy of *PDX1* and rubber tree has two, however only one copy has been identified in rice and bean. We identified only one copy of *PDX2*, 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 (Yost and Lindquist, 1986). Due to altered splicing mechanisms, some genes are differentially spliced under varied environmental stimuli (Akker et al., 2001; Wilson and Cerione, 2000). One example of this affect on splicing in plants is an invertase gene from potato that shows an altered splicing pattern during cold treatment (Bournay et al., 1996).

In summary, we have demonstrated that vitamin $B_6$ 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 $B_6$ is also a strong antioxidant with potential importance during the plant-pathogen defense response.

## Experimental Procedures

### *Superoxide 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 reduction of cytochrome c. The reaction mixture contained a final concentration of 50 mM $KH_2PO_4$, 0.1 mM EDTA, 0.01 mM cytochrome c (Sigma), 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 second intervals for 5 minutes using a Beckman DU 650 spectrophotometer at $OD_{550}$ against a blank

lacking xanthine oxidase. Superoxide dismutase (Sigma) was used as a control. The assay was designed so that 1 unit superoxide dismutase gave approximately 50% inhibition of cytochrome c reduction. Each assay was repeated three times.

*Antioxidant 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 (1978) and Daub (1987). 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 mL of this mixture was combined with 0.2 mL of vitamin $B_6$ stock solutions (final concentrations of 4 μM, 40 μM, 400 μM 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 minute intervals for 90 minutes against a blank lacking β-carotene.

Plant Materials and Growth Conditions

*Nicotiana tabacum* Burley 21 was grown under green house conditions in clay pots or plastic packs in a 4:1 mixture of metro mix and soil with osmocote fertilizer for approximately 8

weeks. One week before experiments, plants were brought to the lab and placed on a plant rack with 100 $\mu mol^{-1}m^{-2}$ light with a 16 hr photoperiod for pathogen tests or in an incubator with 100 $\mu mol^{-1}m^{-2}$ light, 25°C with a 16 hour photoperiod for chemical treatments.

*Chemical treatment*

For salicylic acid treatment, upper and lower sides of leaves were sprayed with 2 mM or 5 mM salicylic acid (Sigma) or with deionized $H_2O$. Leaf tissue from three separate plants was collected at 0, 6, 24, and 48 hours and immediately frozen in liquid nitrogen and stored at -80°C. For methyl jasmonate treatment, upper and lower sides of leaves were sprayed with 1 mM methyl jasmonate (Sigma). After drying for 1 hour, plants were covered with a plastic bag to maintain humidity. Leaf tissue was collected from three separate plants at 24 and 48 hours and immediately frozen in liquid nitrogen and stored at -80°C. qPCR data for methyl jasmonate and salicylic acid treatments are from one and two separate experiments respectively.

*Pathogen treatment*

The terminal half of tobacco leaves were vacuum infiltrated with $10^8$ or $10^9$ cfu/mL of the avirulent bacterium *P. syringae* pv. *phaseolicola* (strain NPS 3121 kindly provided by Peter Lindgren, NC State University) or $10^6$ cfu/mL of the virulent bacterium *P. syringae* pv. *tabaci* (lab stock culture isolated from diseased tobacco in eastern NC). 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 samples per treatment were collected from infiltrated tissue, pooled, frozen in liquid nitrogen, and stored at -80°C. For assessing transcript regulation within and surrounding the HR tissue by quantitative RT-PCR, the lower sides of leaves were infiltrated with $10^8$ cfu/mL *P. syringae* pv. *phaseolicola* using a needleless syringe. Infiltrated regions were marked, and tissue was collected from three individual plants at various time points from the infiltrated region and the 5 mm surrounding region (staying within lateral veins), pooled, frozen in liquid nitrogen, and stored at -80°C (method from Dorey et al., 1997).

*DNA isolation*

Total genomic DNA was extracted using a 3% 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 minutes, extracted twice with 24:1 chloroform:IAA and precipitated with equal volume isopropanol. DNA was washed twice with 70% ethanol and resuspended in water. Samples were RNase treated (Promega, Madison, WI) and stored at -20°C.

*RNA isolation and RT-PCR*

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 RNAsecure (Ambion, Austin, TX) and DNase treated twice with DNA-*free* (Ambion) for 1 hour. RNA was reverse transcribed in a ratio of 20 ng per 1μL 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.

*Gene isolation of PDX1 and PDX2*

Fragments of tobacco *PDX1* and *PDX2* were amplified from Burley 21 cDNA using degenerate primers based on sequence data available from *Arabidopsis*, rice, tomato, rubber tree, yeast, and *Cercospora* created by CODEHOP freeware (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) (Rose et al., 1998). Primers were 5'-GCTGAGAGGTGGTGTTATTATGGAYGTNGT-3' and 5'-CATCATCATCCATATTTCTCAGAACTCKDATRTCNCCC-3' for *PDX1* and 5'-GACCAACATAAACCTACTTGGGGTACNTGYGCNGG-3' and 5'-GATCAATAATTTCTTCAATAACAGGAGCNCKDATRAA-3'for *PDX2*. 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 using the Genome Walker kit (BD Biosciences Clontech, Palo Alto, CA) using primers (IDT, Coralville, IA) 5'-GTAATACGACTCACTATAGGGC-3' and 5'-ACTATAGGGCACGCGTGGT-3' for *PDX1* and a cDNA library (Stratagene, La Jolla, CA) for *PDX2* (T7 primer in combination with primer 5'-TGAGGCCCGCACAAGTACCCCAAGTAG-3' for the 5' end and primer 5'-GCCTCGATTGTACCGTCCACCGAAACT-3' for the 3' end [IDT]). Based on the amplified sequences, primers were designed to isolate full length cDNA and genomic sequences for *PDX1* (5'-ATGGCCGGAAGCGGTGTGGTAA-3', 5'-TCACTCAGAACGATTAGCAT-3') and *PDX2* (5'-ATGGTTGTGGGGGTTCTTGCTTTACAGGGATCTTTCAAC-3' and 5'-CTATTGGTATATGGGAA-3' [IDT]). Sequences were analyzed using vector NTI (Informax of Invitrogen, Carlsbad, CA) and GenBank (NCBI) (Altschul et al., 1990).

*Quantitative PCR*

Gene-specific PCR 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). Primers (Sigma) used were 5'-CTCTCGCCGACGATGAGAAC-3' and 5'-TTACGGCAGCCACAGACAAA-3' for *PDX1,* 5'-CAAATAAAGCAACTGGGCAGAA-3' and 5'-CGGTGGACGGTACAATCGA-3' for *PDX2,* 5'-TGCAGGGTTCCCACTTTCC-3' and 5'-GACGAGCTAAAGGCCGTGTT-3' for *PAL* and 5'-TGGGATTTGTTCTCTTTTCAC-3'

and 5'-TACCTGGAGGATCATAGTTGC-3' for *PR-1a.* For normalization, all genes were compared to expression of 18s ribosomal RNA using Universal PCR master mix (Applied Biosystems). Vitamin $B_6$ biosynthetic genes were compared to standard curves comprised of gene fragments in pGEM T-Easy vectors (Promega) ranging from 40 ng/mL to 40 fg/mL final concentrations. 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).

*Northern analysis*

Total RNA (20ng) was separated on 1.2% formaldehyde gels and transferred onto nylon membranes (Osmonics, Minnetonka, MN). Hybridization was performed at 68°C using PerfectHyb Plus hybridization buffer (Sigma) and digoxygenin-labelled DNA probes according to manufacturer's directions (Roche, Basel, Switzerland). Probes were generated from portions of *PDX1*, *PDX2* and *PR-1a* from tobacco. Blots were washed under stringent conditions and developed following the digoxygenin-alkaline phosphatase kit (Roche) protocol and visualized on film.

*Bacterial growth curves*

To assay the effect of pyridoxine on bacterial viability *in vitro*, $10^8$ cells per ml of *P. syringae* pv. *phaseolicola* were treated with or without 100 mM pyridoxine. Samples were collected at 1.5, 6, and 24 hrs, and serial dilutions plated on King's B medium (20 g peptone, 15 mL glycerol, 1.5 g $K_2HPO_4$, 1.5 g $MgSO_4 \cdot 6\ H_2O$, 15 g agar, per Liter, pH 7.2) and grown at 28°C; colony counts were made at 48 hours. For *in planta* growth assays, the terminal half of tobacco leaves were infiltrated with $10^8$ or $10^9$ *P. syringae* pv. *phaseolicola* or $10^6$ *P. syringae* pv. *tabaci* with or without 100 mM pyridoxine. Leaf discs (7.63 $cm^2$) were collected from three separate plants per time point (1.5, 6, and 24 hours). Leaf discs were surface sterilized in 10% bleach for 5 minutes followed by 70% ethanol for 10 minutes. 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 determined at 48 hrs and the cfu/$cm^2$ leaf area determined.

*Vitamin $B_6$ bioassay*

Total vitamin $B_6$ was extracted from lyophilized tobacco leaf tissue using a modified protocol from Gregory (1982). Three to six plants were assayed individually for each treatment. After grinding lyophilized tissue in liquid nitrogen, 0.1 g tissue powder was mixed with 30 mL 0.44N HCL and autoclaved at 121°C for 4 hours. The pH was brought to 4.8 using sodium

acetate. 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 was then brought up to a 50 mL volume and filtered through miracloth and then filter sterilized using a 50 mL steriflip unit (0.22uM) (Millipore, Bedford, MA). Extracts were stored in the dark at -20°C until use.

Yeast (*S. cerevisiae*) strain 9080 (ATCC Center) was grown overnight at 30°C/ 220 rpm in 40 mL pyridoxine Y medium (5.3 g/100 ml pyridoxine YM [Becton Dickinson, Franklin Lakes, NJ]) amended with 40 ng pyridoxine after autoclaving. Overnight cultures were washed twice with pyridoxine Y medium to remove residual pyridoxine. Assay tubes (14 mL) were set-up to contain $2x10^8$ yeast cells in 4 mL of medium with 50 μL of leaf extract. Standards contained 0, 2, 4, 6, and 8 ng total pyridoxine per 4 mL volume. All samples and standards were run in triplicate. Tubes were incubated for 16 hrs at 30°C/220 rpm. Growth was measured using a Beckman DU 650 spectrophotometer at $OD_{540}$. The vitamin $B_6$ content of the samples was determined based on comparison to the standard curve.

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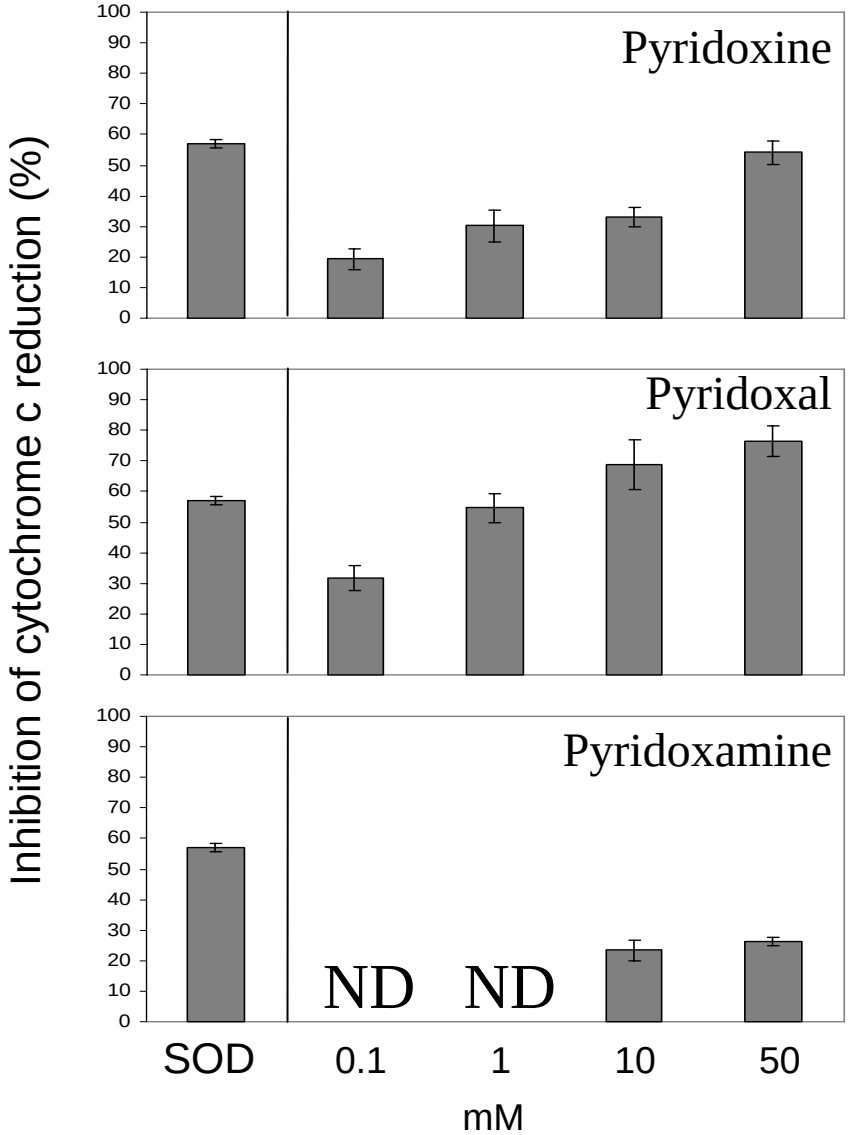



**Figure 1.** Quenching of superoxide by $B_6$ vitamers pyridoxine, pyridoxal, and pyridoxamine, measured as the percent inhibition of cytochrome c reduction. Superoxide dismutase (SOD) control represents 1 unit SOD. Error bars represent standard error of three replicates. (ND=not done)

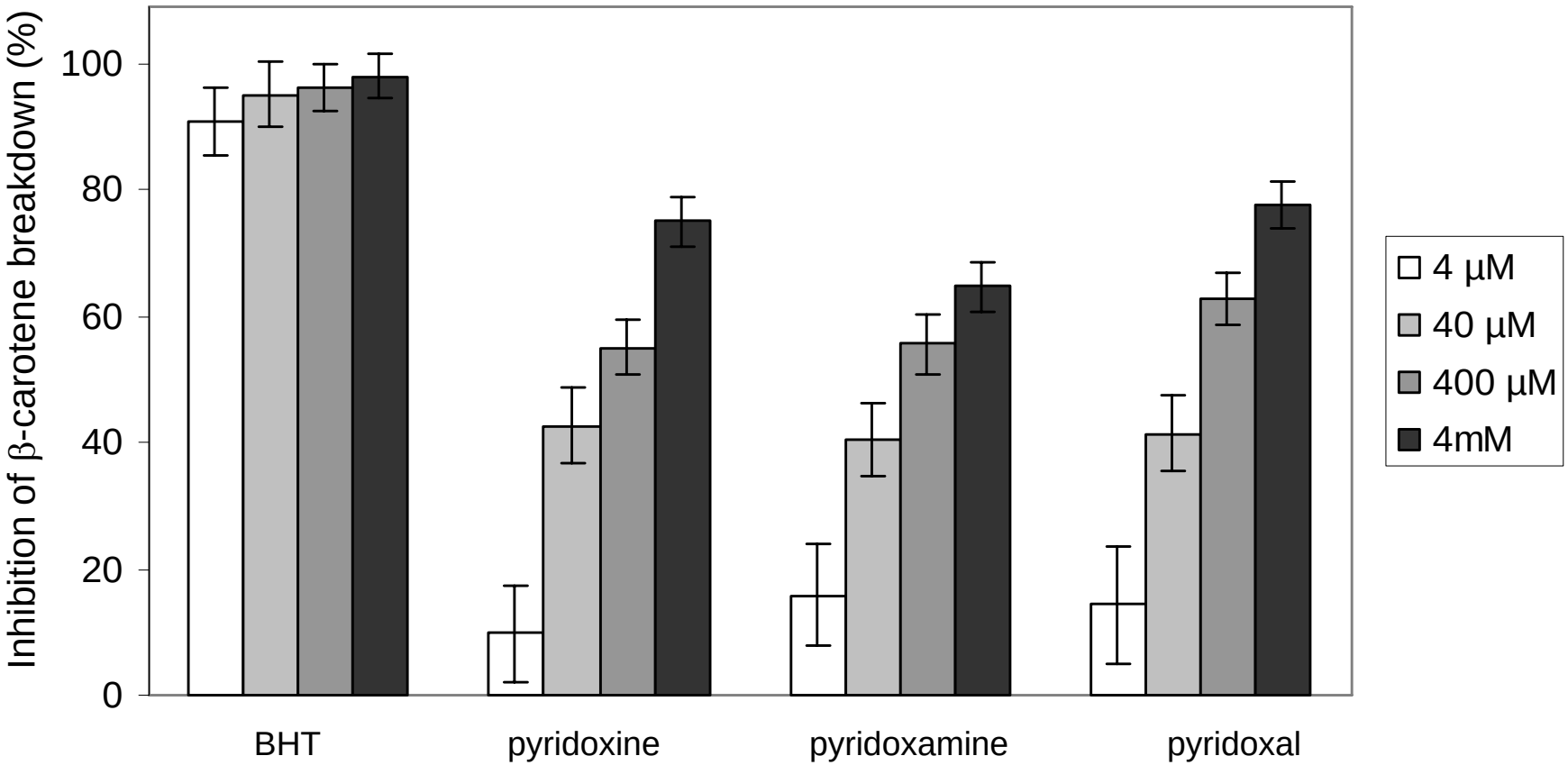



**Figure 2.** Antioxidant activity of $B_6$ vitamers determined by measuring the inhibition of β-carotene breakdown caused by the coupled oxidation of β-carotene and linoleic acid. Butylated hydroxytuluene (BHT), a strong phenolic antioxidant, was used as a control for antioxidant activity. Error bars represent standard error of three replicates.

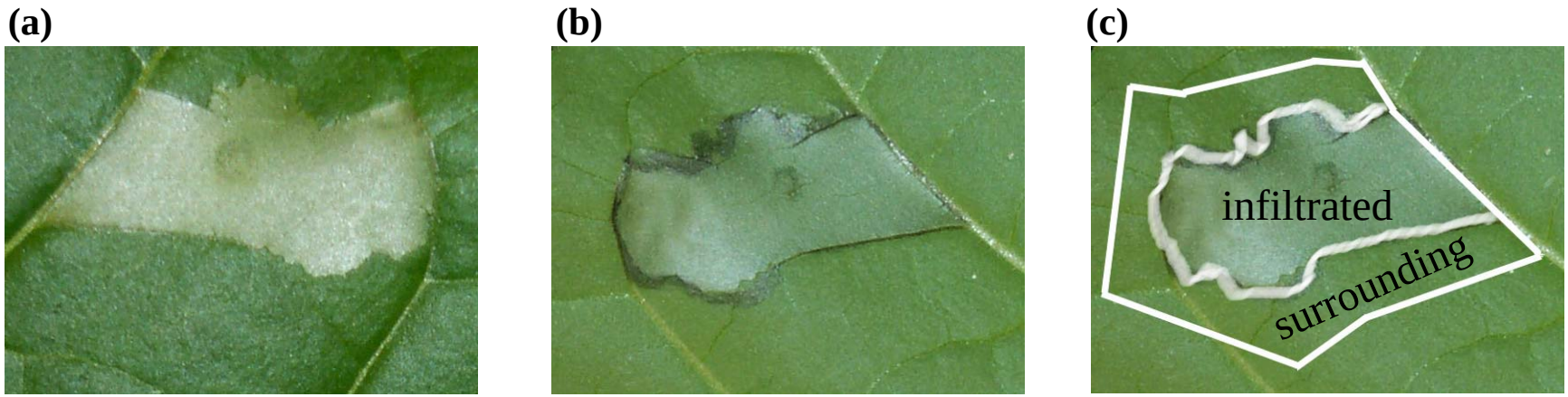



**Figure 3.** (a) Front and (b and c) back of a tobacco leaf showing a hypersensitive response after needleless syringe infiltration of the avirulent pathogen *Pseudomonas syringae* pv. *phaseolicola*. Infiltrated region was outlined with a sharpie marker on the backs of leaves. (c) We tested separately the infiltrated regions and the 5 mm surrounding regions, staying within major lateral veins, for transcript abundance.

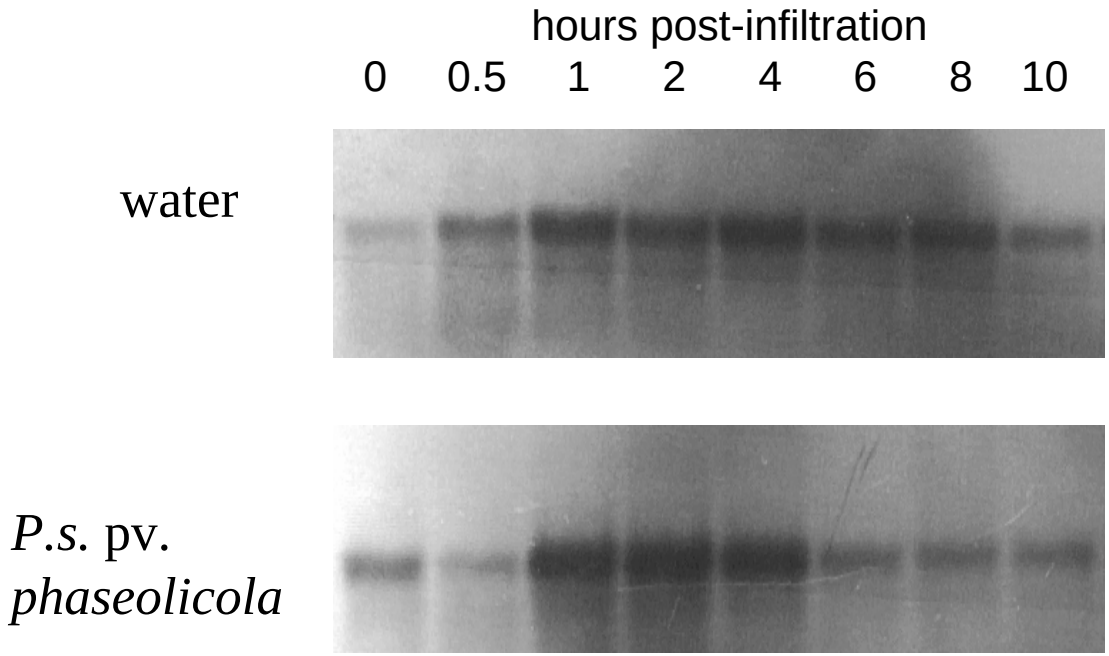



**Figure 4.** Northern analysis assessing *PDX1* transcript accumulation following vacuum infiltration of tobacco leaves with the avirulent pathogen *Pseudomonas syringae* pv. *phaseolicola* or water control.

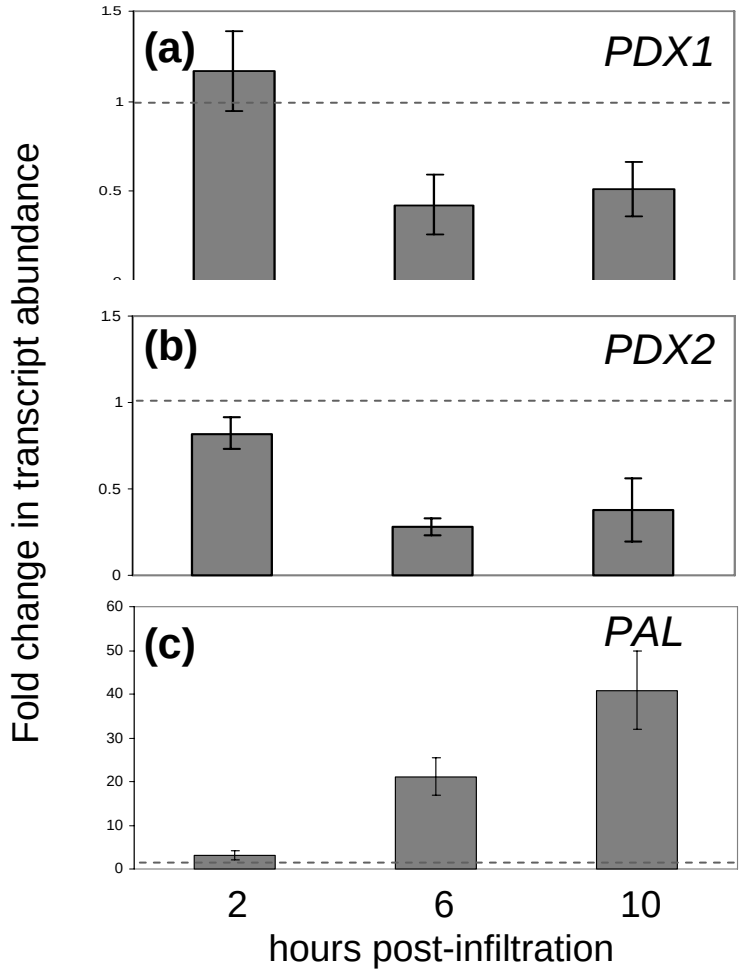



**Figure 5.** Fold change in transcript abundance of (a) *PDX1*, (b) *PDX2,* and *(c) PAL* in tobacco leaf tissue infiltrated with the avirulent bacterium *P. syringae* pv. *phaseolicola*. Transcript abundance was measured by quantitative RT-PCR and normalized to 18s expression. Data are shown as the 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.

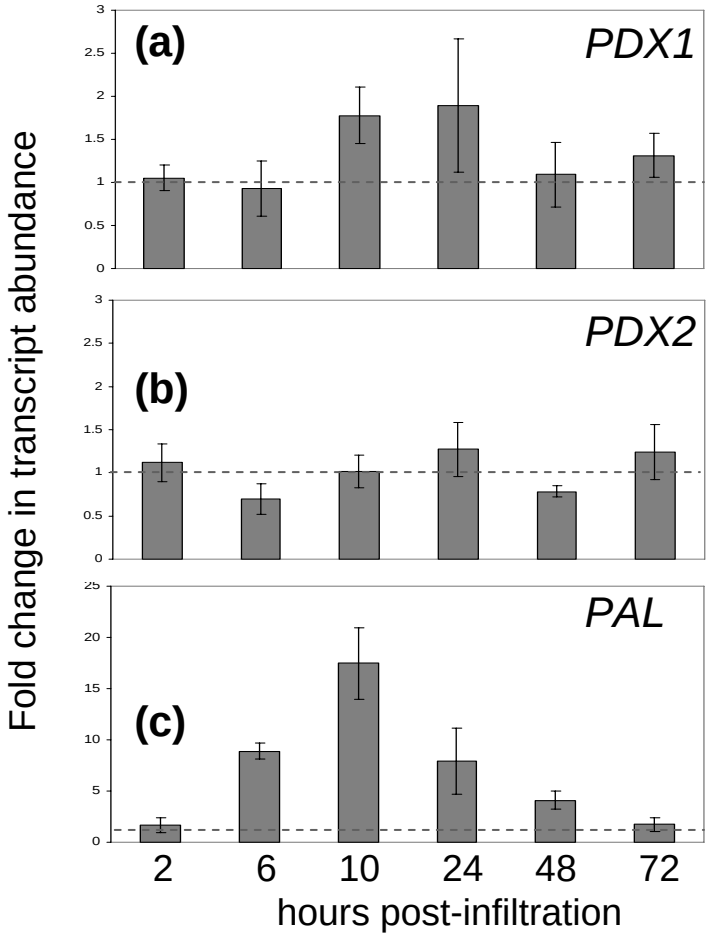



**Figure 6.** Transcript abundance of (a) *PDX1*, (b) *PDX2,* and (c) *PAL* in 5 mm tobacco leaf tissue surrounding the region infiltrated with the avirulent bacterium *P. syringae* pv. *phaseolicola*. Transcript abundance was measured by quantitative RT-PCR and normalized to 18s expression. Data are 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.

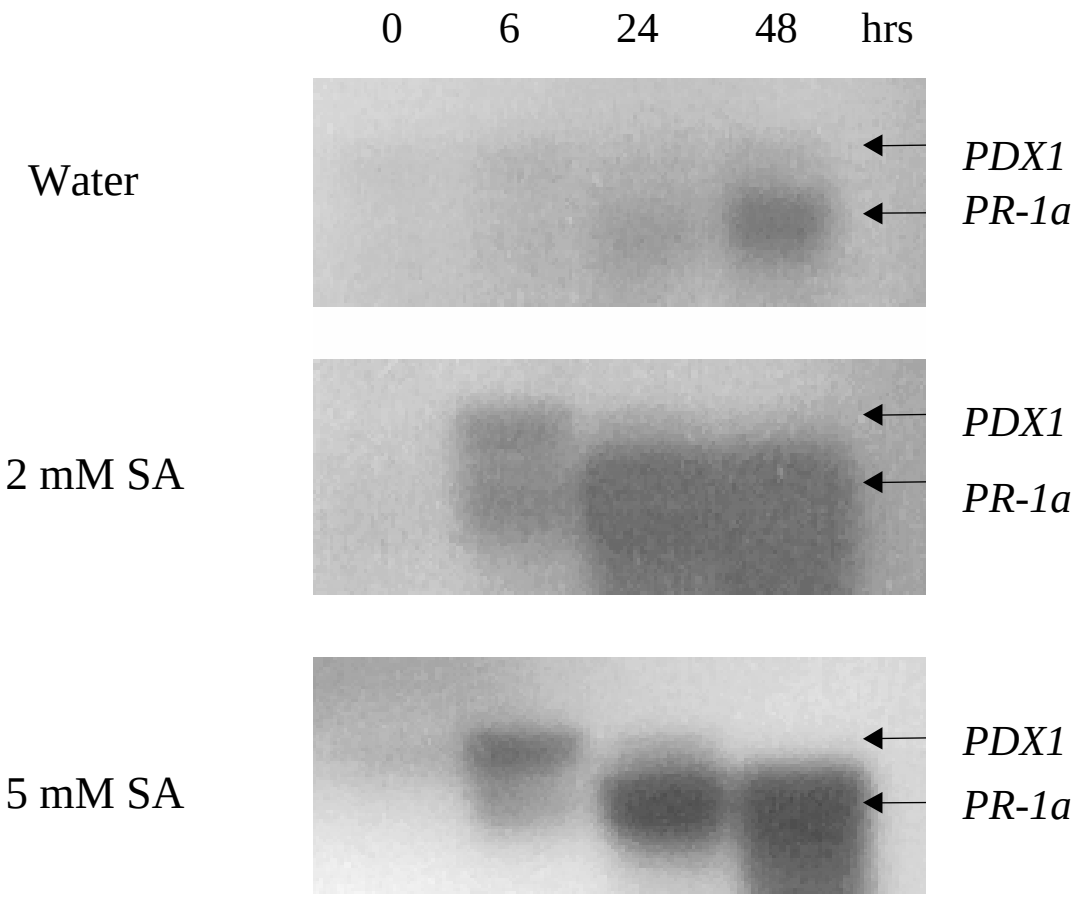



**Figure 7.** Northern analysis of tobacco leaf tissue from plants sprayed with 2 mM or 5 mM salicylic acid or water. Blots probed with digoxigenin-dUTP labeled *PDX1* and *Pr-1a* (included as an SAR control).

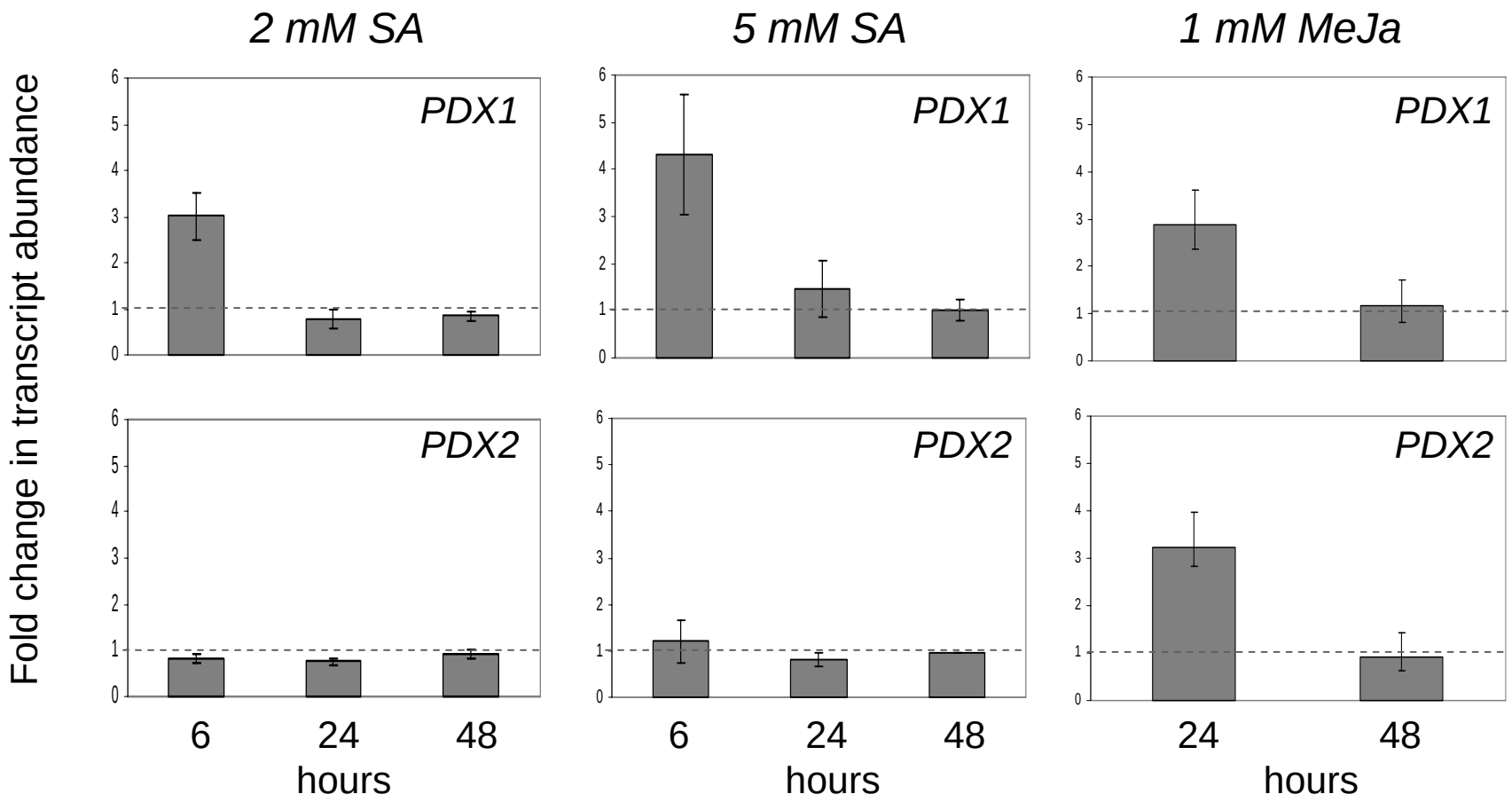



**Figure 8.** Transcript abundance of *PDX1* and *PDX2* in tobacco leaf tissue sprayed with 2 mM or 5 mM salicylic acid or 1 mM methyl jasmonate measured by qRT-PCR and normalized to 18s expression. Transcript abundance was measured by qRT-PCR and normalized to 18s expression. Salicylic acid data are shown as the average fold change of transcript from pathogen-treated plants over water control-treated plants from two experiments. Methyl jasmonate data are the average of three qRT-PCR replicates from one experiment. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. Error bars=standard error.

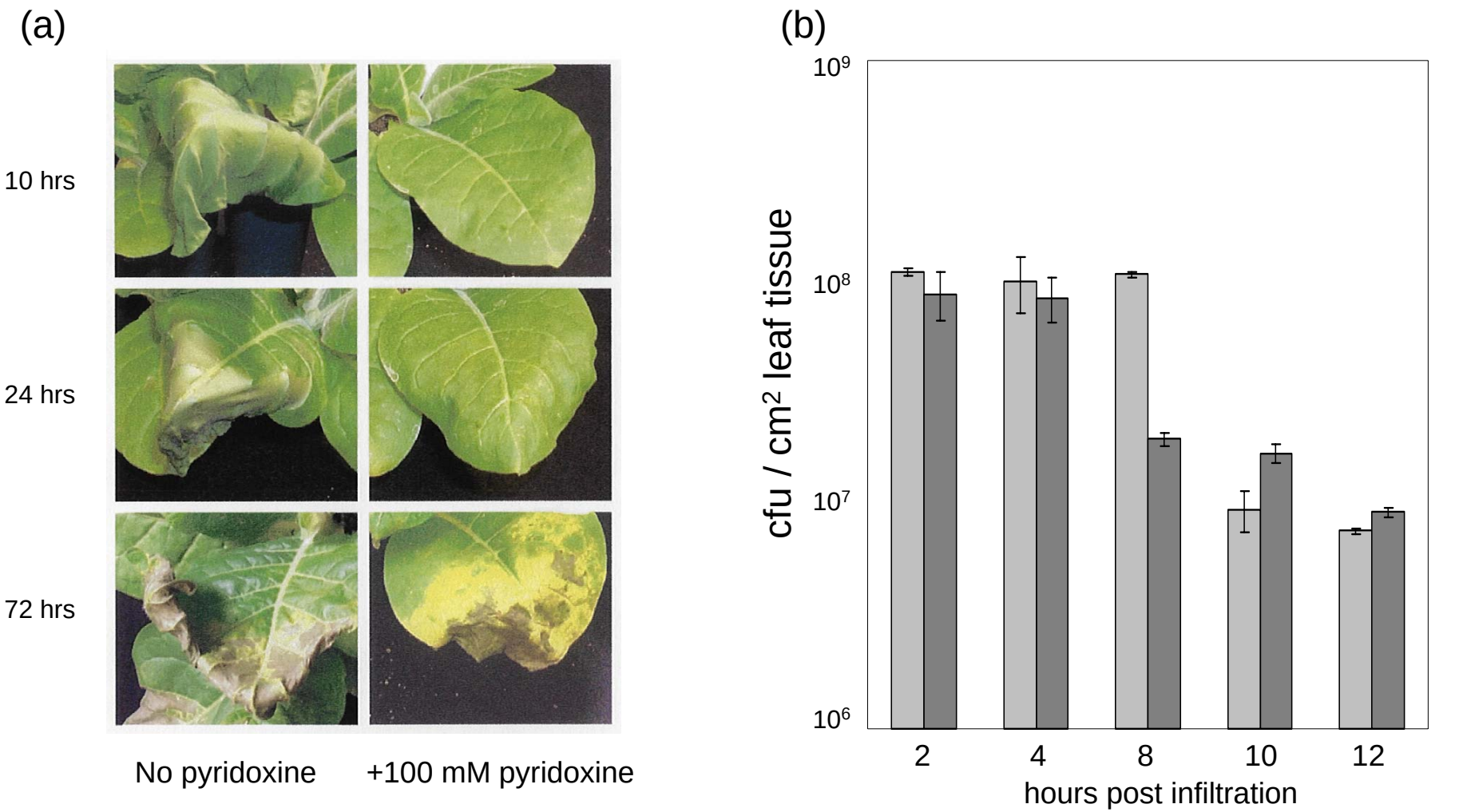
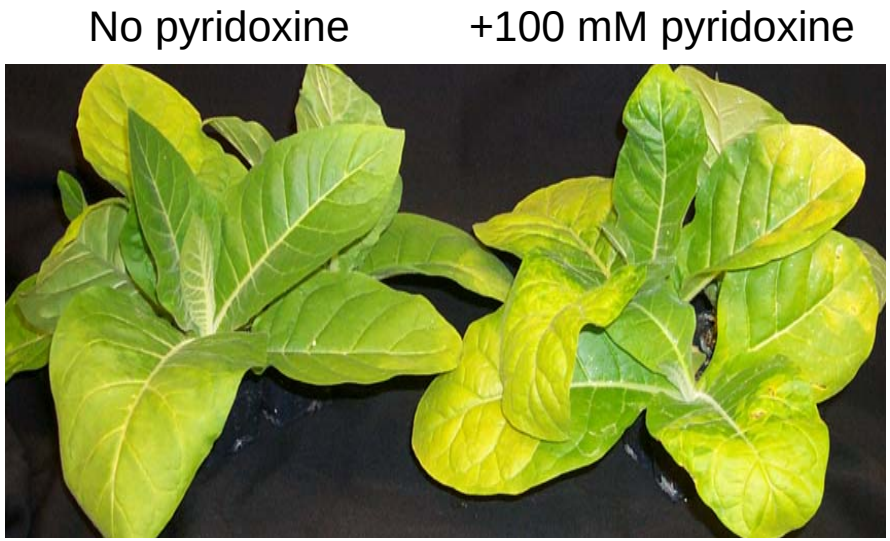



**Figure 9.** (a) Hypersensitive defense response in tobacco plants infiltrated with $10^9$ cfu/ml of the avirulent bacterium *Pseudomonas syringae* pv. *phaseolicola* with and without 100 mM pyridoxine. (b) *In planta* assay measuring cfu of *P. syringae* pv. *phaseolicola*/cm$^2$ leaf tissue in absence (light grey bars) or presence (dark grey bars) of 100 mM pyridoxine. Error bars represent standard deviation of three replicates.

(a)




(b)

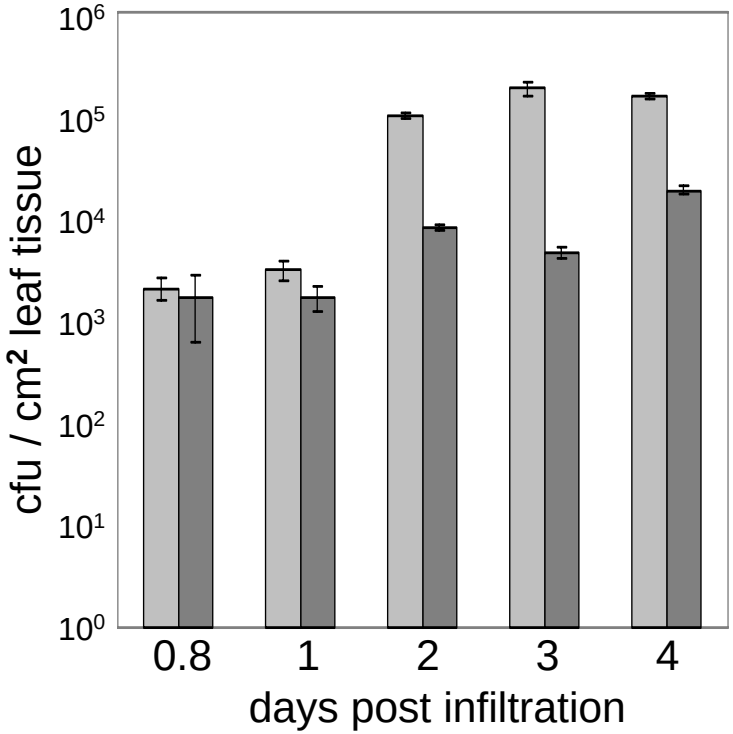



**Figure 10.** (a) Effect of 100 mM pyridoxine on disease symptoms in tobacco plants infiltrated with $10^6$ cfu/ml of the virulent pathogen *Pseudomonas syringae* pv. *tabaci*. (b) *In planta* assay measuring cfu of *P. syringae* pv. *tabaci*/cm$^2$ leaf tissue in absence (light grey bars) or presence (dark grey bars) of 100 mM pyridoxine. Error bars represent standard deviation of three replicates.

# Chapter 3

## VITAMIN $B_6$ BIOSYNTHETIC GENES IN *ARABIDOPSIS THALIANA* AND THEIR DIFFERENTIAL REGULATION DURING ABIOTIC STRESS

**VITAMIN $B_6$ BIOSYNTHETIC GENES IN *ARABIDOPSIS THALIANA* AND THEIR DIFFERENTIAL REGULATION BY ABIOTIC STRESS**

## Abstract

Plants contain an alternate pathway for vitamin $B_6$, distinct from the well characterized pathway in *Escherichia coli*. As this is a newly discovered pathway, little is known about $B_6$ production in plants. In addition to this new information on $B_6$ biosynthesis, vitamin $B_6$ has recently been associated in diverse organisms with conditions of cellular oxidative stress, whether through regulation studies of biosynthetic genes or through studies of the $B_6$ molecules themselves. We used *Arabidopsis thaliana* to study the role of vitamin $B_6$ during abiotic stress. We identified vitamin $B_6$ biosynthetic genes (*PDX1* and *PDX2*) from *A. thaliana* by sequence homology. We found three full-length copies of *PDX1* and one full-length copy of *PDX2*. The upstream regions of these genes were found to contain several potential stress-responsive transcription factor binding sites. Quantitative RT-PCR showed differential regulation to abiotic stressors including high light, chilling, drought, and ozone. As these stressors affect specific organelles or regions within a cell with greater impact, this disparity in *PDX1* regulation may correlate with a differential cellular localization for the protein products of the three *PDX1*'s.

## Introduction

Vitamin $B_6$, in its active form pyridoxal 5'-phosphate, is an essential cofactor most notable for its involvement in amino acid biosynthesis. The *de novo* biosynthetic pathway for vitamin $B_6$ has been fully characterized in *Escherichia coli* (Arps and Winkler, 1987; Dempsey, 1969; Drewke and Leistner, 2001; Hill and Spenser, 1986; Hill and Spenser, 1996; Hockney and Scott, 1979; Lam et al., 1992; Lam and Winkler, 1990; and Lam and Winkler, 1992; Man et al., 1996; Roa et al., 1989; Schoenlein et al., 1989; Zhao and Winkler, 1996). Recently, however, an alternate *de novo* $B_6$ biosynthetic pathway was identified. This alternate pathway is actually the more prevalent pathway for $B_6$-producing organisms, common to plants, archaebacteria, fungi and most bacteria (Mittenhuber, 2001). The two proteins unique to this pathway, PDX1 and PDX2, have just this year been ascribed a function. PDX2 was confirmed to be a glutaminase through enzymatic studies (Dong et al., 2004) and through deciphering the crystal structure (Bauer et al., 2004). Through complementation studies with *E. coli* mutants, we have shown PDX1 can catalyze the formation of the pyridoxine ring (Wetzel et al., 2004). Thus, PDX2 most likely supplies a nitrogen containing precursor that PDX1 incorporates into the pyridoxine ring.

In addition to this new-found knowledge on $B_6$ biosynthesis, recent information has also uncovered that vitamin $B_6$, similar to vitamins C and E, not only catalyzes enzymatic reactions, but also shows antioxidant activity. Both metabolic and gene regulation studies have connected vitamin $B_6$ with cellular oxidative stress.

Chemical assays have shown that $B_6$ vitamers are potent quenchers of singlet oxygen and superoxide and can prevent oxidative damage to fatty acids (Denslow et al., unpublished). This corroborates evidence from animal models associating higher vitamin $B_6$ levels with decreased levels of active oxygen species (AOS) and less oxidative damage to macromolecules. In blood assays, vitamin $B_6$ had three times the antioxidant activity of vitamin C and was shown to quench superoxide production (Stocker et al., 2003; Jain and Lim, 2001; Kannan and Jain, 2004). In rabbit lens cells, vitamin $B_6$ prevented protein oxidation, a cause of cataract formation (Jain et al., 2002). Vitamin $B_6$-deficient rats showed elevated inflammatory responses and lipid peroxidation (Lakshmi et al., 1991). And finally, clinical trials have shown that vitamin $B_6$ supplements prevent or delay eye and nerve damage associated with diabetes and attributed to superoxide production (Jain and Lim, 2001).

Gene regulation studies in numerous and diverse organisms have also linked vitamin $B_6$ to oxidative stress through studies identifying the *de novo* biosynthetic genes (*PDX1* and *PDX2*) or $B_6$ scavaging pathway genes (pyridoxal kinase, oxidase, and reductases) and their products as being affected by oxidative stress conditions. In the bacterium *Bacillus subtilis*, the PDX1 homologue shows increased expression during treatment with the superoxide generator paraquat (Antelmann et al., 1997); hydrogen peroxide treatment of *Schizosaccharomyces pombe* led to increased transcript abundance of *PDX2* and pyridoxal reductases (M. W. Toone, Paterson Institute for Cancer Research, Manchester, UK, personal communication); and *Saccharomyces cerevisiae* shows increased transcript and protein accumulation in one of the three sets of *PDX1* and *PDX2* at entry into stationary phase, a time of high oxidative stress (Braun et al., 1996; Padilla

et al., 1998). Vitamin $B_6$ pathway genes also show regulation during conditions leading to oxidative stress in plants. In *Arabidopsis thaliana*, UV-B radiation led to increased transcript of one of three *PDX1's* (Brosche et al., 2002) and *PdxK* (pyridoxal kinase) has been implicated in salt tolerance and shows increased transcript during cold stress (Shi et al., 2002). In *Phaseolis vulgaris*, abscisic acid and wounding led to increased *PDX1* transcript (Graham et al., 2004). And, salicylic acid and ethylene, chemical inducers of plant-pathogen defense response, a process involving high oxidative stress, have led to increases in *PDX1* transcript in *Hevea brasiliensis* (rubber tree) (Sivasubramaniam et al., 1995).

While plants produce AOS as by-products of normal processes including photosynthesis, photorespiration, and metabolism, during times of stress, the levels of AOS often reach damaging levels. To counteract the detrimental effects associated with increased AOS during abiotic stress, cells employ a host of compounds and enzymes including antioxidant enzymes such as superoxide dismutases, catalases, ascorbate peroxidase, and glutathione reductase, antioxidant molecules including reduced glutathione, ascorbate, a-tocopherol, flavonoid pigments, and carotenoids, and proteins with repair functions (heat shock proteins) (Dat et al., 2000; Davison et al., 2002; Grene , 2002; Havaux and Kloppstech, 2001; Mittler, 2002; Winkel-Shirley, 2002).

As the *de novo* vitamin $B_6$ has not been well characterized in plants and $B_6$ has been shown to have antioxidant activity, we wanted to explore the *de novo* vitamin $B_6$ pathway in *A. thaliana* and determine if it is linked to oxidative stress conditions produced during abiotic stress. One of the *de novo* biosynthetic genes, *PDX1*, had been identified on chromosome 3 in *A. thaliana* through sequence identity (Ökrész et al.,

1998), however no further studies have been published on this pathway in *A. thaliana*. Here we show that *A. thaliana* ecotype Columbia contains three full-length copies of *PDX1* and one copy of *PDX2*. In the upstream regions of these four genes, we identified potential transcription factor binding sites associated with responsiveness to stress. Quantitative reverse transcription PCR (qRT-PCR) showed differential regulation to abiotic stressors including high light, chilling, drought, and ozone, suggesting that vitamin $B_6$ plays an important role in oxidative stress responses in plants.

## Results

### *Identification of $B_6$ biosynthetic genes in A. thaliana ecotype Columbia*

We identified homologues to vitamin $B_6$ biosynthetic genes, *PDX1* and *PDX2,* in *A. thaliana* ecotype Colombia by sequence homology to known *PDX1* and *PDX2* sequences from the fungi *Cercospora nicotianae* and *S. cerevisiae* using Genbank and TAIR databases (Altschul et al., 1990; Rhee et al., 2003). Three full-length copies of *PDX1* were identified, one each on chromosomes 2, 3, and 5 (*PDX1(2)*, *PDX1(3)*, and *PDX1(5)* respectively). The locus number for each identified gene is shown in Table 1. *PDX1(2)* and *PDX1(5)*, the more similar of the three homologues, each encode proteins of 309 amino acids in length that are 89% identical. *PDX1(3),* which had been identified previously through sequence identity (Ökrész et al., 1998), encodes a slightly longer

protein of 314 amino acids, with an extended amino terminus from PDX1(2) and PDX1(5). PDX1(3) is only 60% identical to PDX1(2) and PDX1(5). All three *PDX1* genes have open reading frames composed of one exon, as is found for all *PDX1*'s identified to date (Braun et al., 1996; Denslow et al., unpublished; Ehrenshaft et al., 1998; Osmani et al., 1999). A partial copy of *PDX1* of 240 nucleotides was also identified on chromosome 2. It is 100% identical to the first third of *PDX1(2)* and is located 4 kb away. We identified one copy of *PDX2* found on chromosome 5. *PDX2* contains an open reading frame made from 6 exons and encodes for a protein of 255 amino acids.

*Stress-associated promoter elements*

As we are interested in the role of pyridoxine in oxidative stress, the 1.4 Kb upstream regions for the *PDX1*'s and *PDX2* were evaluated for the presence of motifs connected to stress-related gene expression (Table 1). All four biosynthetic gene promoters contained potential binding sites for members of the WRKY, CCAAT-enhancer binding proteins (C/EBP), MYB, and MYC transcription factor families, involved in gene regulation under different stresses including pathogen associated stress, salicylic acid treatment, and wounding (WRKY), cellular stress and heat shock (C/EBP), and drought, cold and abscisic acid (MYB and MYC). Other stress-related regulatory element motifs, including abscisic acid responsive element (ABRE), C repeat/drought responsive element (C repeat/DRE), ethylene responsive element (ERE), G box, and TGA binding sites, were found in some, but not all of the upstream regions of the four $B_6$

biosynthetic genes. These elements are associated with gene expression due to osmotic stress and drought (ABRE), osmotic stress, drought and cold, independent of absicisic acid (C repeat/DRE), ethylene, jasmonic acid, pathogen attack, and ozone stress (ERE), light, oxygen deprivation, and treatment with the hormones abcscisic acid, ethylene, and methyl jasmonate (G box), and pathogen attack and salicylic acid treatment (TGA). The presence of the promoter elements is consistent with regulation of these genes under stress conditions.

*$B_6$ biosynthetic gene regulation during abiotic stress*

Due to the presence of stress-responsive elements in promoter regions of the $B_6$ biosynthetic genes, we tested the effect of various environmental and oxidative stresses on transcript abundance. For high light stress, plants grown in a growth chamber under 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light were transferred to 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light and transcript accumulation in rosettes was measured after 1, 4, and 7 days of stress treatment using qRT-PCR. Results are shown in Figure 1, where transcript abundance is shown as fold change of high light-treated plants over control plants that were maintained at 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light. *PDX1(2)*, *PDX1(3)*, *PDX1(5)*, and *PDX2* all showed a similar pattern in transcript accumulation reaching the highest fold change over controls early in the stress treatment (day one), followed by a return to control levels or below on day four, and then another, smaller increase above control levels on day seven of treatment (Figure 1A). Although all four genes showed the same pattern of response, the response on day

one for *PDX1(3)* was significantly less than that of the other three genes, reaching a maximum 1.5-fold change over control. By day 7, high light-treated plants were red in color, due to accumulation of anthocyanins in response to the high-light stress (Figure 1B).

To test the effect of chilling stress on transcript accumulation of $B_6$ biosynthetic genes, plants grown in a chamber at 20°C were switched to a 5°C chamber. Transcript abundance in rosettes was determined by qRT-PCR on days 1, 4, and 7 of stress treatment and compared to that of control treated plants maintained at 20°C. Results are shown in Figure 2. An initial increase in transcript abundance on day one of treatment was observed for all four $B_6$ biosynthetic genes followed by a continuous decline back to control levels on days four and seven (Figure 2A). *PDX1(2)* showed the greatest increase in transcript abundance compared to control levels with an average 8-fold change. *PDX1(5)* and *PDX2* also showed large fold changes after one day of cold stress. Again, similar to what was seen with light stress, *PDX1(3)* showed the least amount of change compared to control treatments, reaching only an average 1.8-fold increase on day one of treatment. Chilling stress visibly affected the plants causing reduced growth and increased anthocyanin production (Figure 2B).

Plants were drought stressed by withholding water. Symptoms of drought stress were not dramatic, with no observable symptoms at 7 days and a slight reduction in growth at 14 days (Figure 3B). After seven days of drought stress all four genes showed no change or a slight increase in transcript abundance compared to control plants. By 14 days, *PDX1(2), PDX1(5),* and *PDX2,* but not *PDX1(3),*showed a slight, but significant

increase in transcript abundance compared to controls of about 1.8-fold (Figure 3A). Transcript abundance for *PDX1(3)* was unaffected, remaining near control levels.

We also tested the direct effect of reactive oxygen species by treating *A. thaliana* with ozone and hydrogen peroxide. For ozone treatment, plants were exposed to 8 hours per day of 125 ppb ozone or air control for 1 and 2 days. qRT-PCR from rosettes showed three distinct responses (Figure 4). For both time points, *PDX1(2)* transcript abundance decreased, with transcript levels dipping to half that found in control plants after one day of treatment (Figure 4). By contrast, *PDX1(3)* showed a consistent increase in transcript abundance compared to controls, reaching a 3-fold increase on day 1 of ozone treatment. *PDX1(5)* and *PDX2* showed a similar response, distinct from the other two genes, with little change after 1 day of treatment and a slight decrease after 2 days (Figure 4).

Hydrogen peroxide treatment (1 mM) of *A. thaliana* leaves had little to no effect on transcript abundance of any of the four $B_6$ biosynthetic genes 0.5 hours after treatment, but all four showed a slight increase 3 hours after treatment (Figure 5). *PDX1(5)* showed the most change in transcript abundance from control with an average 2.2-fold change three hours after treatment, followed by *PDX1(2)* with an average 1.6–fold change in transcript abundance over control 3 hours after treatment. *PDX1(3)* and *PDX2* showed the least change, both with an average 1.3–fold change in transcript abundance over control at three hours after treatment.

## Discussion

The PDX1/PDX2 *de novo* vitamin $B_6$ biosynthetic pathway found in plants, fungi, archaebacteria, and most bacteria, was only recently discovered and shown to be distinct from the well characterized pathway in *E. coli* (Arps and Winkler, 1987; Dempsey, 1969; Drewke and Leistner, 2001; Hill and Spenser, 1986; Hill and Spenser, 1996; Hockney and Scott, 1979; Lam et al., 1992; Lam and Winkler, 1990; and Lam and Winkler, 1992; Man et al., 1996; Roa et al., 1989; Schoenlein et al., 1989; Zhao and Winkler, 1996). Given the recent discovery, little is known about the pathway or the homologues in plants. Using sequence homology, we identified *de novo* vitamin $B_6$ biosynthetic genes in *A. thaliana* ecotype Columbia. Three full-length *PDX1* homologues and one full length *PDX2* homologue were identified. All three *PDX1* genes were composed of one uninterrupted exon, as is true for all *PDX1*'s identified to date, suggestive of a need to ensure fast and efficient translation. All three *PDX1* homologues were strongly conserved, as are all *PDX1* homologues thus far identified. One of the *A. thaliana PDX1*'s (*PDX1(3)*), however, is more distinct from the others, showing less sequence identity and an elongated amino terminus.

As vitamin $B_6$ has been shown to quench AOS and protect molecules from oxidative damage, we characterized the promoter regions of the four *A. thaliana* genes for elements associated with stress related gene expression. We found potential binding sites for WRKY, CCAAT-enhancer binding proteins, MYB, MYC, abscisic acid responsive, C repeat/drought responsive, ethylene responsive, G box, and TGA

MYC transcription factor families have been found to activate gene expression in response to drought, cold and abscisic acid (Abe et al., 1997; Abe et al., 2003; Chinnusamy et al., 2003; Pastori and Foyer, 2002; Urao et al., 1993). CCAAT-enhancer binding protein (C/EBP) motifs are found in numerous eukaryotes and have roles in gene regulation in response to hormones, inflammation and cellular stress (Ramji and Foka, 2002). In plants, the C/EBP elements, in combination with other elements, have been associated with the expression of heat shock genes (Haralampidis et al., 2002; Rieping and Schoffl, 1992). The abscisic acid responsive element (ABRE) is associated with gene expression due to osmotic stress and drought (Shinozaki et al., 2003). The C repeat/drought responsive element (C repeat/DRE) also promotes gene expression in response to high osmoticum, drought and cold, but its function is independent of abscisic acid (Pastori and Foyer, 2002; Yamaguchi-Shinozaki and Shinozaki, 1994). The ethylene responsive element (ERE) leads to gene induction by ethylene and jasmonic acid, as well as by pathogen attack (Gutterson and Reuber, 2004). The ERE has also been implicated in gene response to ozone stress (Grimmig et al., 2003). The G box element, working in conjunction with other cis-acting regulatory elements, is associated with gene expression under diverse environmental stimuli such as light, oxygen deprivation, and treatment with the hormones abcscisic acid, ethylene, and methyl jasmonate (Menkens et al., 1995; Vranová et al., 2002). TGA factors are associated with gene expression during pathogen attack and salicylic acid treatment (Durrant and Dong, 2004). The presence of these regulatory elements is consistent with data that point to gene regulation of vitamin $B_6$ biosynthetic genes during stress conditions.

Given the presence of so many stress-associated promoter elements, we assessed the transcriptional regulation of *de novo* vitamin $B_6$ biosynthetic genes during oxidative stress causing abiotic stress. Abiotic stresses, such as high light, low temperature, drought, salt, nutrient deprivation, ozone and UV irradiation all lead to an increased cellular oxidative stress (Fryer et al., 2002; Grene, 2002; Karpinsiki et al. 1999; Sweetlove et al., 2002). For the stressors we tested, including high light, chilling, drought, ozone and hydrogen peroxide, the three *PDX1*'s and *PDX2* differed in their transcript accumulation patterns. Again, *PDX1(3)* diverged the most in its response pattern from the other *PDX1*'s and *PDX2*. While all four genes showed increased transcript abundance during high light and chilling stress, the increase for *PDX1(3)* was much lower. Drought stress led to little if any change in *PDX1(3)* transcript abundance, while showing an increase for the other *PDX1*'s and *PDX2*. And, while ozone stress led to a 3-fold increase in *PDX1(3)* transcript abundance, no increase was observed for *PDX1(2)*, *PDX1(5)* or *PDX2*. *PDX1(2)* even showed a striking decrease in transcript abundance after one day of ozone stress.

The vast differences in regulation of the different genes under the various abiotic stresses was not expected. It is important to note that while all of these stressors lead to conditions of cellular oxidative stress, each stress has a primary site of impact that is more directly affected. Chloroplasts and mitochondria produce AOS during the stresses that impact or interfere with the electron transport chains of photosynthesis and metabolism. Environmental conditions such as high light, temperature extremes, drought, salinity, or nutrient stress, can hinder electron transport, forcing electrons to reduce oxygen, producing superoxide. Chloroplasts have an added source of AOS as excess

excitation energy can energize oxygen, producing singlet oxygen as well (Fryer et al., 2002; Grene, 2002; Karpinsiki et al. 1999; Sweetlove et al., 2002). Other stresses, while still potentially affecting chloroplasts and mitochondria, primarily impact other sites within the cell. Ozone stress, for example, causes oxidative damage directly and is thought to primarily impact the apoplast (Grene, 2002). Ozone stress has also been shown to activate a plasma membrane localized NADPH-oxidase, creating superoxide and, through dismutase activity, hydrogen peroxide in the apoplast (Overmeyer et al., 2003). Thus, while there is overlap, various types of abiotic stresses lead to the increased production of AOS in specific regions within the cell.

Perhaps due to the organelle-specific production of AOS by different stressors, antioxidants found in these organelles have been reported to show stress-specific regulation. A chloroplast-localized superoxide dismutase (FSD2) responds to high light and UV, but does not respond to ozone (Grene, 2002 and references within). Other studies have found, using northern analysis, that chloroplast-localized Fe superoxide dismutases and glutathione reductase showed decreased transcript abundance after ozone exposure while the cytosolic antioxidants Cu-Zn superoxide dismutase, glutathione-S-transferase, ascorbate peroxidase and a neutral peroxidase increase after ozone stress (Sharma and Davis 1994; Conklin and Last, 1995). Mn superoxide dismutases, found in mitochondria and peroxisomes, have been reported to respond to salt, chilling, and drought, but not to high light (Grene , 2002 and references within).

It is still unknown in any system, including plants, where vitamin $B_6$ is synthesized in the cell or where PDX1 and PDX2 proteins localize. We hypothesize that the differential response of *PDX1(3)* may reflect a different cellular localization of the

gene product as compared to the products of *PDX1(2)*, and *PDX1(5).* We are currently conducting GFP gene fusion experiments with the *PDX* biosynthetic genes in order to determine this information.

In summary, we have identified *de novo* vitamin $B_6$ biosynthetic genes, *PDX1* and *PDX2*, in *A. thaliana.* Stress-related regulatory elements were identified in the promoter regions of these genes. Consistent with the presence of stress-related promoter elements in the upstream regions of these biosynthetic genes, qRT-PCR studies showed that they are regulated, though differentially, by stress induced through high light, chilling, drought, ozone and hydrogen peroxide treatments, conditions which lead to cellular oxidative stress. These results support the importance of vitamin $B_6$ antioxidant activity during stress *in planta*.

## Materials and Methods

### *Sequence identification and analysis*

Homologues to *PDX1* and *PDX2* were identified in *A. thaliana* ecotype Colombia by sequence homology to known *PDX1* and *PDX2* sequences from *C. nicotianae* and *S. cerevisiae* using blast searches through NCBI and TAIR databases (Altschul et al., 1990; Rhee et al., 2003). Sequences were analyzed using Vector NTI and PLACE searches (Higo et al., 1999). Motifs used: ABRE: YACGTGGC; C/EBP: CCAAT; C/DRE:

CCGAC; ERE: GCCGCC; G box: CACGTG; MYB: WAACCA, YAACKG, and AMCWAMC; MYC: CANNTG; TGA: TGACG; WRKY: TTGACY.

*Plant growth and stress treatment*

*A. thaliana* ecotype Columbia (Lehle Seed, Round Rock, TX) was grown on *Arabidopsis* growth medium (Lehle seed) at 20ºC, 8 hour photoperiod (8AM-4PM), 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light in a Percival growth chamber. Plants were watered 2-3 times per week, fertilizing every two weeks with half-strength Miracle-Gro. Plants were stressed at 5 to 6 weeks of age. Drought and hydrogen peroxide experiments were conducted in the Percival chambers (conditions above). For drought treatment, water was withheld from plants for two weeks with samples collected at days 7 and 14. For hydrogen peroxide treatment, leaves were sprayed with either water or 1 mM hydrogen peroxide and samples collected at 0.5 and 3 hours. High light, chilling and ozone experiments were conducted in growth chambers in the Southeastern Plant Environment Laboratory (NCSU Phytotron). Plants were moved to control chambers (20°C, 8 hour photoperiod (8AM to 4PM), 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light) two weeks prior to experiment. High light experiments were conducted at 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light and chilling experiments were conducted at 5°C. For ozone stress, plants were exposed to 125 ppb ozone or air control (in collaboration with Kent Burkey). For all treatments, plants were transferred to treatment chambers (if applicable) at 4 PM on day 0 and samples were taken at the appropriate day at 3:30 PM. Controls were collected at the same time points as stress treated plants. For

each treatment / time point, rosettes from 3 plants were collected and combined and immediately frozen in liquid nitrogen. Tissue was stored at -80°C for RNA extractions.

*RNA isolation and RT-PCR*

Total RNA was extracted from leaf tissue using Tri-Reagent (Sigma) following manufacturer's suggested protocol and adding a second phenol-chloroform extraction. RNA samples were resuspended in RNAsecure (Ambion, Austin, TX) and DNase treated twice with DNA-*free* (Ambion) for 1 hour. RNA was reverse transcribed in a ratio of 20 ng per 1µL 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.

*Quantitative PCR*

PCR was performed using 5 µL cDNA mix (corresponding to 100 ng starting total RNA) in 25 uL total volume of reaction mix containing SYBR Green mastermix (Applied Biosystems) and gene specific primers (0.8 pmol/uL final concentration). Primers used (IDT, Coralville, IA): PDX1(2) forward primer: 5'-TCTCCCTTCTCCGTGAAAGTTG-3', PDX1(2) reverse primer: 5'-GCGTTGACGACATCCATGATT-3'; PDX1(3) forward primer: 5'-

AGGTCGGATTAGCTCAGGTACTTC-3', PDX1(3) reverse primer: 5'-CGGATTCAGCGAGCTTAGCTT-3'; PDX1(5) forward primer: 5'-TTTGCGGTTGCCGGAAT-3', PDX1(5) reverse primer: 5'-ATCATCGCCGCACCTTCA-3'; PDX2 forward primer: 5'-GTTCATACGTGCTCCAGCTGTT-3', PDX2 reverse primer: 5'-TTGATGGGACGGGATAATCC-3'. For normalization, all genes were compared to expression of 18s ribosomal RNA (Applied Biosystems) using taqman mastermix and 1 μL cDNA (20 ng starting total RNA). All reactions were done in triplicate. Quantitative PCR reactions were carried out on an MJ Research DNA Engine Opticon2 or an Applied Biosystems ABI7000 sequence detection system (parameters: 50°C for 2', 95°C for 10', followed by 45 cycles of 95°C for 15s, 60°C 1').

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**Table 1. *De novo* vitamin $B_6$ biosynthetic genes in *A. thaliana* ecotype Columbia and potential binding sites for stress-responsive regulatory elements.** Motifs were identified in the 1.4 Kb region upstream of ATG. Number identified for each motif is shown in parentheses.

| **Gene Name** | *PDX1(2)* | *PDX1(3)* | *PDX1(5)* | *PDX2* |
|---|---|---|---|---|
| **Locus** | At2g38230 | At3g16050 | At5g01410 | At5g60540 |
| **Stress Elements** | | | | |
| cold | MYB (4)<br>MYC (3) | C/DRE (1)<br>MYB (8)<br>MYC (9) | C/DRE (1)<br>MYB (4)<br>MYC (4) | MYB (5)<br>MYC (1) |
| drought | ABRE (3)<br>MYB (4)<br>MYC (3) | ABRE (3)<br>C/DRE (1)<br>MYB (8)<br>MYC (9) | C/DRE (1)<br>MYB (4)<br>MYC (4) | MYB (5)<br>MYC (1) |
| high light | | G box (2) | | |
| heat shock | C/EBP (6) | C/EBP (1) | C/EBP (2) | C/EBP (3) |
| osmotic stress | ABRE (3) | ABRE (3)<br>C/DRE (1) | C/DRE (1) | |
| ozone | | | ERE (2) | ERE (1) |
| pathogen stress | TGA (1)<br>WRKY (2) | TGA (4)<br>WRKY (2) | ERE (2)<br>WRKY (2) | ERE (1)<br>WRKY (5) |
| wounding | WRKY (2) | WRKY (2) | WRKY (2) | WRKY (5) |
| absicisic acid | ABRE (3)<br>MYB (4)<br>MYC (3) | ABRE (3)<br>G box (2)<br>MYB (8)<br>MYC (9) | MYB (4)<br>MYC (4) | MYB (5)<br>MYC (1) |
| ethylene | | G box (2) | ERE (2) | ERE (1) |
| jasmonic acid | | G box (2) | ERE (2) | ERE (1) |
| salicylic acid | TGA (1)<br>WRKY (2) | TGA (4)<br>WRKY (2) | WRKY (2) | WRKY (5) |

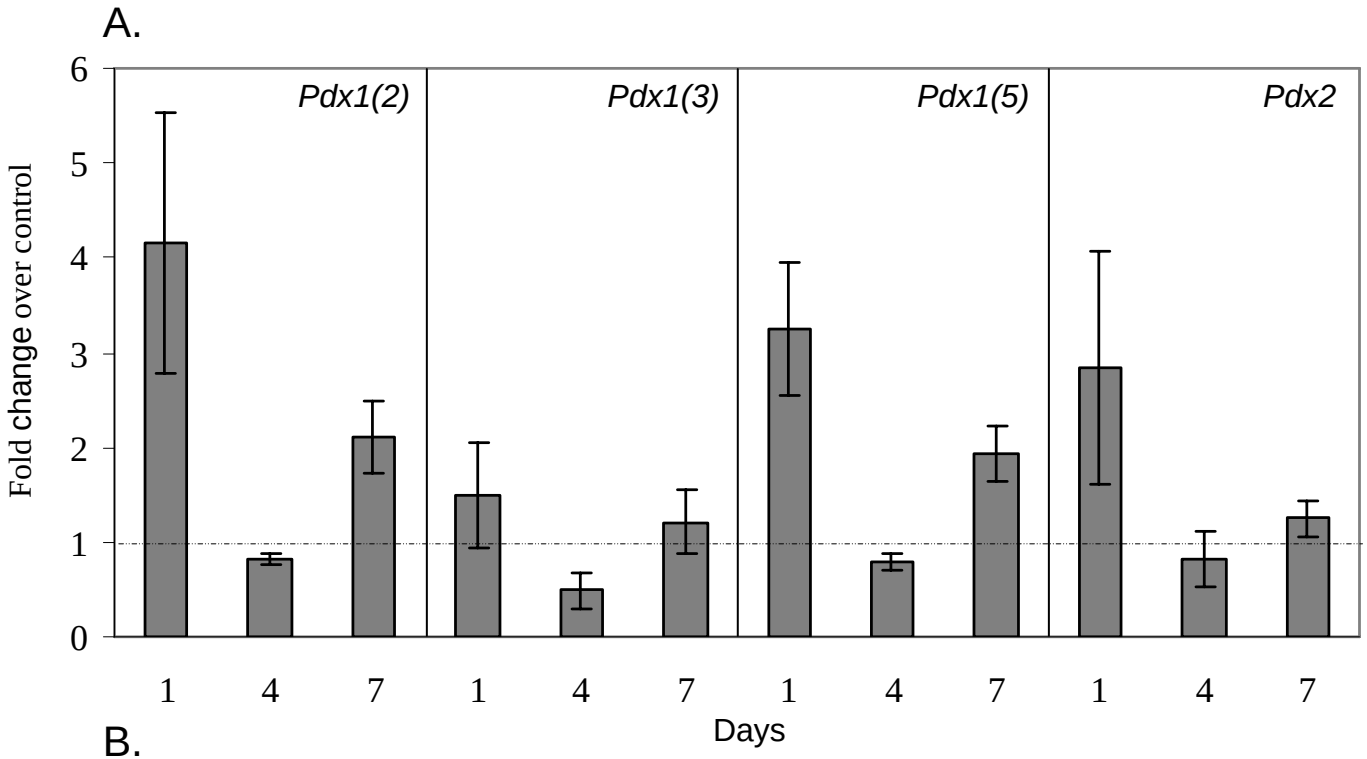


**Figure 1.** A) Quantitative RT-PCR showing transcript abundance of $B_6$ biosynthetic genes in *A. thaliana* rosettes after increasing light strength from 200 to 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ for 1, 4, and 7 days. Transcript abundance shown as the average fold change in light stressed plants (1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light) over control treated plants (200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light) from five experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples are normalized to 18s. Error bars=standard error.
B) *Arabidopsis* plants after 7 days of control treatment (200 $\mu mol \cdot s^{-1} \cdot m^{-2}$) (left) and high light treatment (100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) (right). Purple color is due to increased production of anthocyanins.

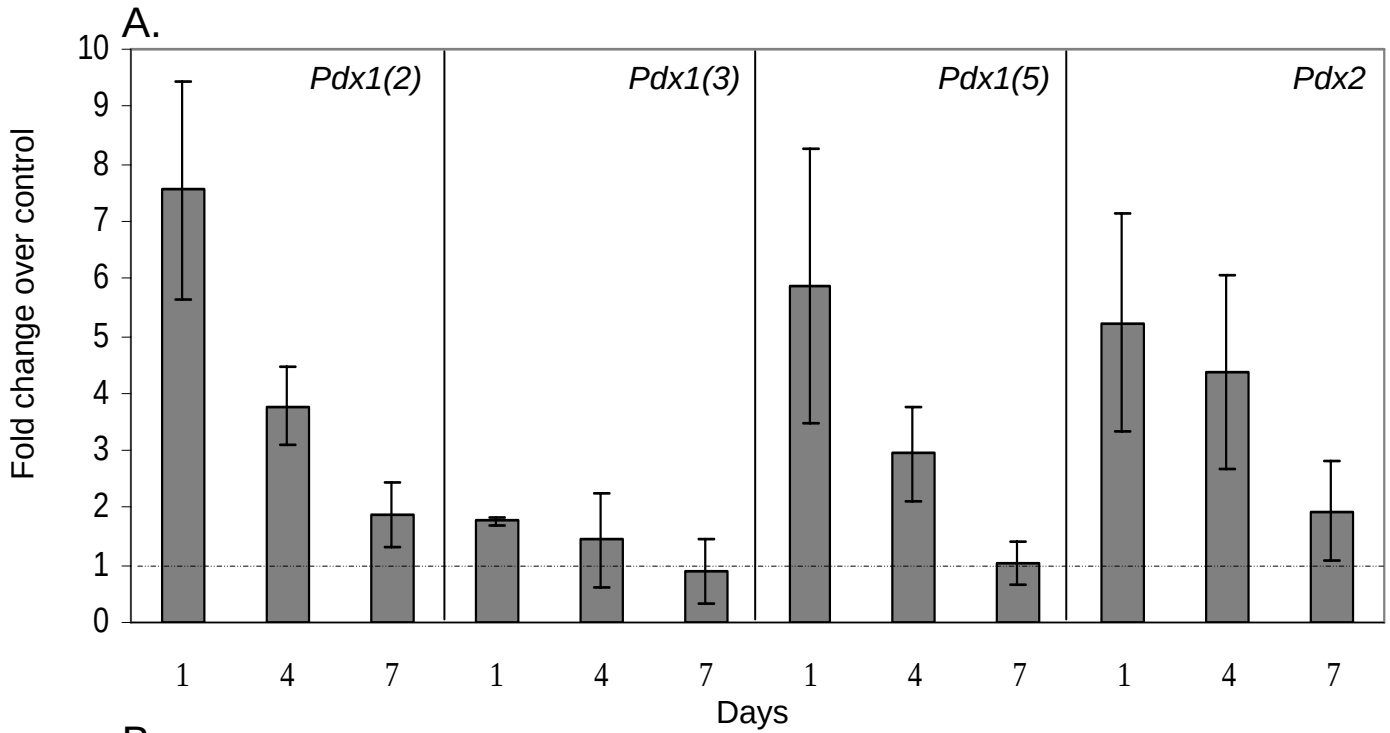


**Figure 2.** A) Quantitative RT-PCR showing transcript abundance of $B_6$ biosynthetic genes in *A. thaliana* rosettes after decreasing temperature from 20°C to 5°C for 1, 4, and 7 days. Transcript abundance shown as the average fold change in chilling stressed plants (5°C) over control treated plants (20°C) from five experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples are normalized to 18s. Error bars=standard error.

B) *A. thaliana* plants after 7 days of control treatment (20°C) (left) and chilling treatment (5°C) (right).

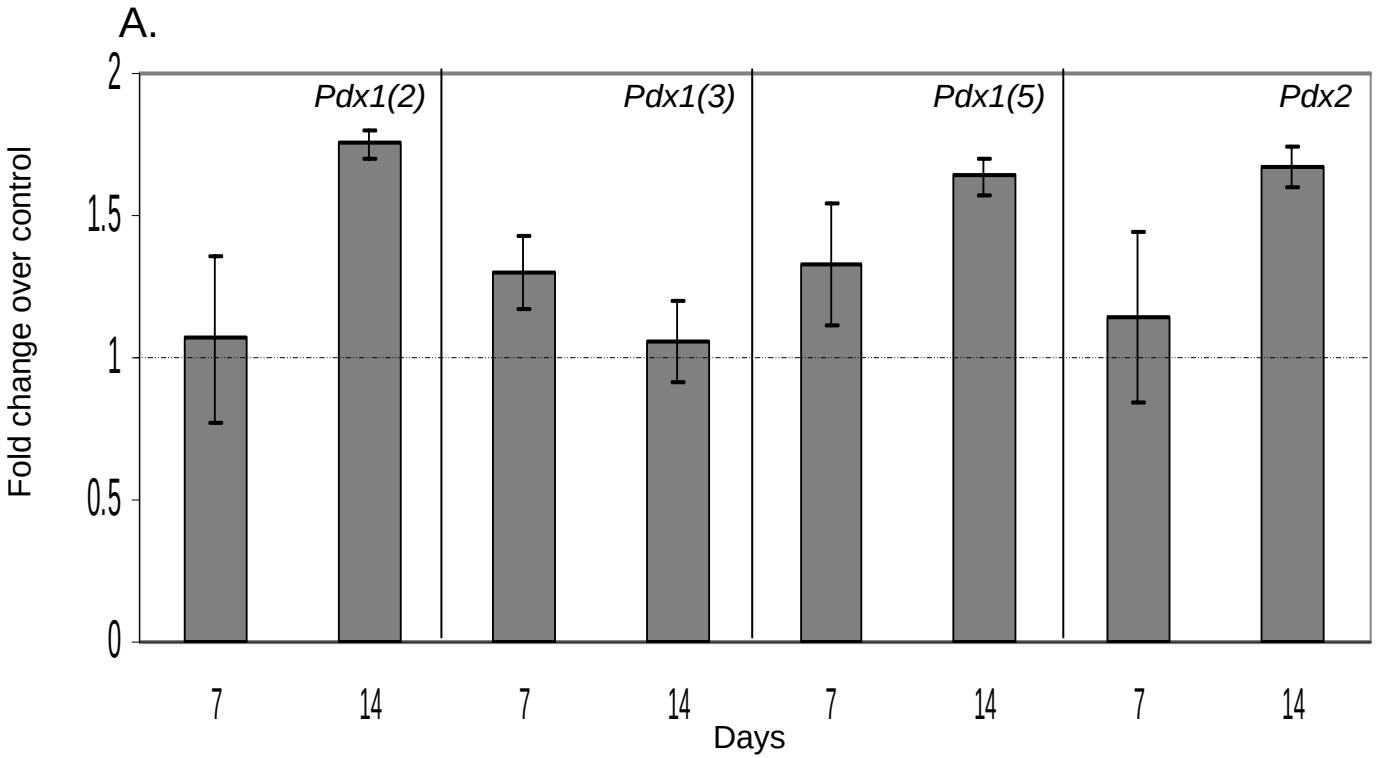


**Figure 3.** A) Quantitative RT-PCR showing transcript abundance of $B_6$ biosynthetic genes in *A. thaliana* rosettes after withholding water for 7 and 14 days. Transcript abundance shown as the average fold change in drought stressed plants (no water) over control treated plants (watered) from three experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples are normalized to 18s. Error bars=standard error.
B) *A. thaliana* plants after 14 days of control treatment (water) (left) and drought treatment (no water) (right).

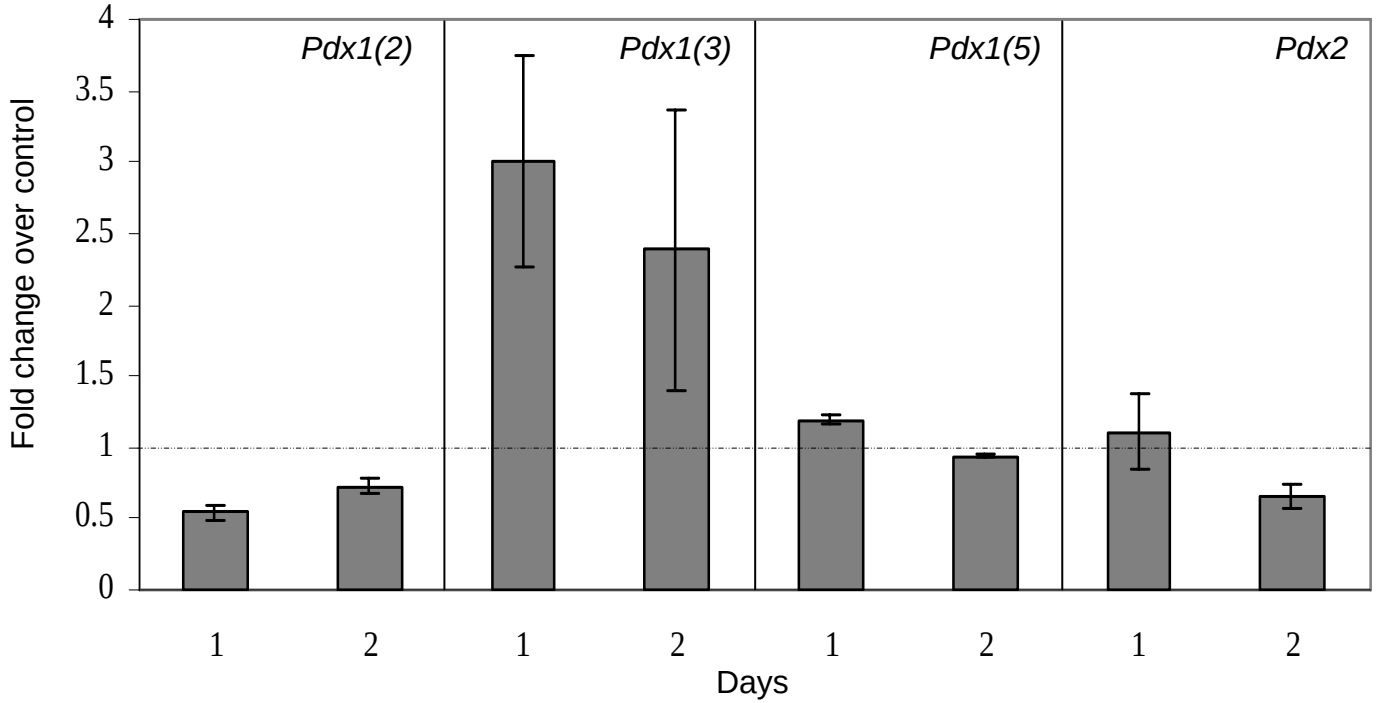


**Figure 4.** A) Quantitative RT-PCR showing transcript abundance of $B_6$ biosynthetic genes in *A. thaliana* rosettes after 1 and 2 days of ozone treatment (125 ppb). Transcript abundance shown as the average fold change in ozone stressed plants over control treated plants from three experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples are normalized to 18s. Error bars=standard error.

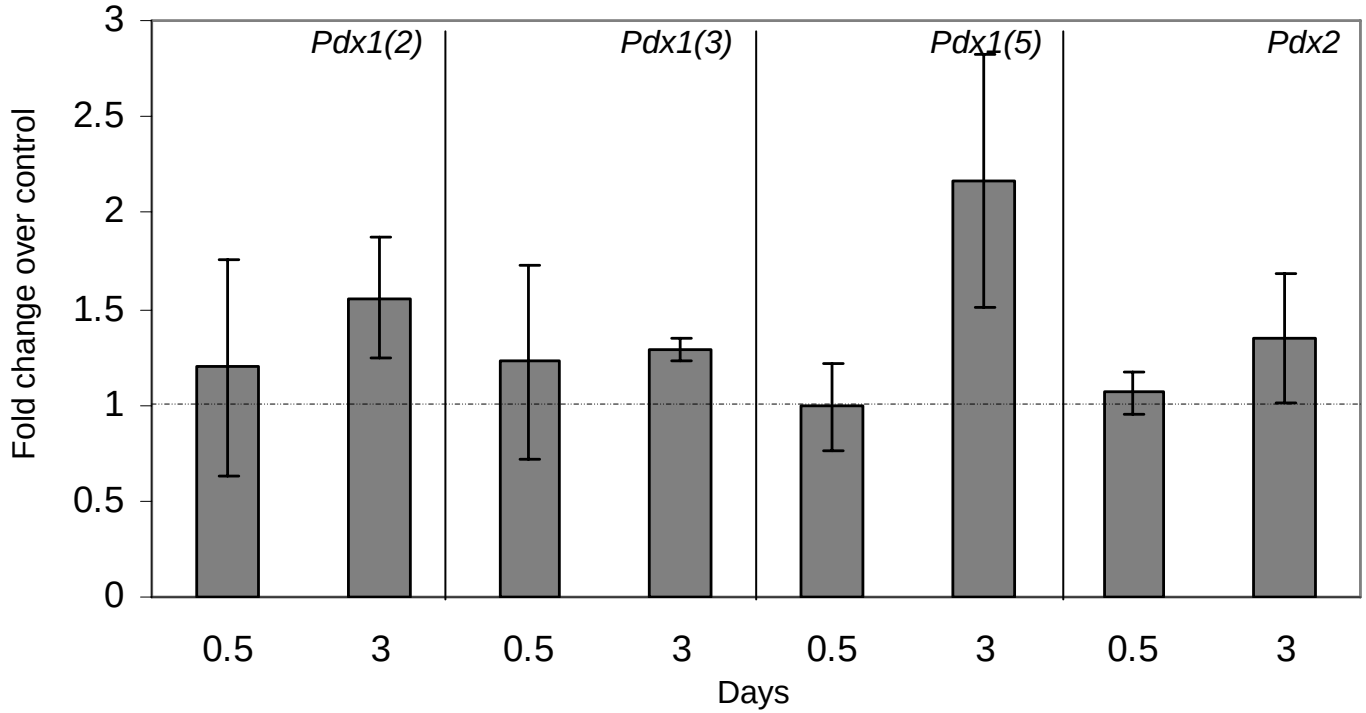


**Figure 5.** A) Quantitative RT-PCR showing transcript abundance of $B_6$ biosynthetic genes in *A. thaliana* rosettes 0.5 and 3 hours after spraying with 1 mM hydrogen peroxide. Transcript abundance shown as the average fold change in hydrogen peroxide treated plants over water control treated plants from two experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples are normalized to 18s. Error bars=experimental values from both experiments.

## APPENDIX A: OVER-EXPRESSION OF PDX1 IN TOBACCO TO ASSESS POTENTIAL RESISTANCE TO *CERCOSPORA NICOTIANAE*.

**Objective:** The goal of this study was to over-express PDX1 in tobacco and to test the susceptibility of transformants to the fungus Cercospora nicotianae, the causal agent of frog eye leaf spot on tobacco.

**Procedure:**

Transgenic Tobacco

PDX1 from Cercospora nicotianae was cloned into the binary vector pBI121 either with or without a β-glucuronidase fusion. Expression was driven by the cauliflower mosaic virus 35s promoter and terminated with the nolapine synthase terminator. pBI121 contains a kanamycin resistance cassette. For plant transformation, plasmids were put into *Agrobacterium tumafaciens* strain EHA105 using the freeze-thaw method. Plasmids were selected for with kanamycin (50 μg/mL). The EHA105 strain of agrobacterium was selected for using rifampicin (25ug/mL). Tobacco leaf discs (*Nicotiana tabacum* cv. Burley 21) were transformed by the *Agrobacterium* using the leaf disc dip method. Transformed plants were selected by growth on 50 μg/mL kanamycin and were verified through Southern analysis.

Pathogen Resistance Assessment

Transformed tobacco plants were tested for their resistance to *C. nicotianae* through greenhouse inoculation studies. *C. nicotianae* was grown on V-8 medium in lighted incubator at 22°C for 5 days to induce sporulation. Spores were collected using a paint brush in sterile water and diluted to a concentration of $5x10^4$ spores/mL. Using a vacuum pump, spores were sprayed on the backs of fully expanded tobacco leaves just until inoculum began to drip off. Two leaves per plant were tested. The entire tobacco plant was then covered with a plastic bag and placed in the shade for two days to prevent plants from overheating. Bags were then removed and plants returned to sunlight. Symptoms were assessed 14-17 days after inoculation. Plants were given a rating from 0 to 4 (0= no lesions; 1= less than 50 lesions per leaf with no coalescence; 2= 50-200 lesions per leaf with no coalescence; 3= >200 lesions per leaf with coalescence <1cm; 4= >200 lesions per leaf with coalescence >1cm). Resistance was defined as a rating of 2 or less. PDX1 transgenics were compared to vector control transgenic controls and wild type controls.

**Results:**

For PDX1-GUS transformants, 46 transgenics were tested for Cercospora resistance. Of these, 2 plants showed resistance in greenhouse trials. Resistance was no longer found in the progeny of these plants.

For PDX1 transformants, 72 transgenics were tested for Cercospora resistance, with none showing resistance.

## APPENDIX B: CHANGES IN TRANSCRIPT ABUNDANCE OF *PDX1(2)*, *PDX1(5)*, AND *PDX2* IN ARABIDOPSIS OVER A DAY.

**Objective:** The goal of this study was to determine if transcript abundance levels changed for vitamin $B_6$ biosynthetic genes throughout a day.

**Procedure:**

*Arabidopsis thaliana* ecotype Columbia were grown in a Percival incubator at 20°C, with an 8 hour photoperiod (9AM-5PM), and approximately 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light. At 6 weeks of age, plants were sampled at time points throughout the day to monitor transcript levels. Sample times were every 4 hours during the dark cycle and every 2 hours during the light cycle. Samples were also collected 10 minutes before and after the start of the light cycle and the end of the light cycle. Rosettes from three plants were collected for each time point. RNA was extracted using TriReagent (Sigma). Quantitative RT-PCR was performed using SYBR Green (Applied Biosystems).

**Results:**

Figure 1 shows changes in transcript abundance for the vitamin $B_6$ biosynthetic genes over the course of a day. Transcript abundance is shown as percent of maximum value over the experiment. The three genes tested do show variations in transcript abundance

throughout the day. All three genes show an increase in transcript accumulation 2 hours after the lights come on, followed by a drop in transcript levels. This drop continues for *PDX1(2)*. *PDX1(5)* and *PDX2*, however, show a second increase in levels, reaching their maximum values at 6 hours after the lights come on.

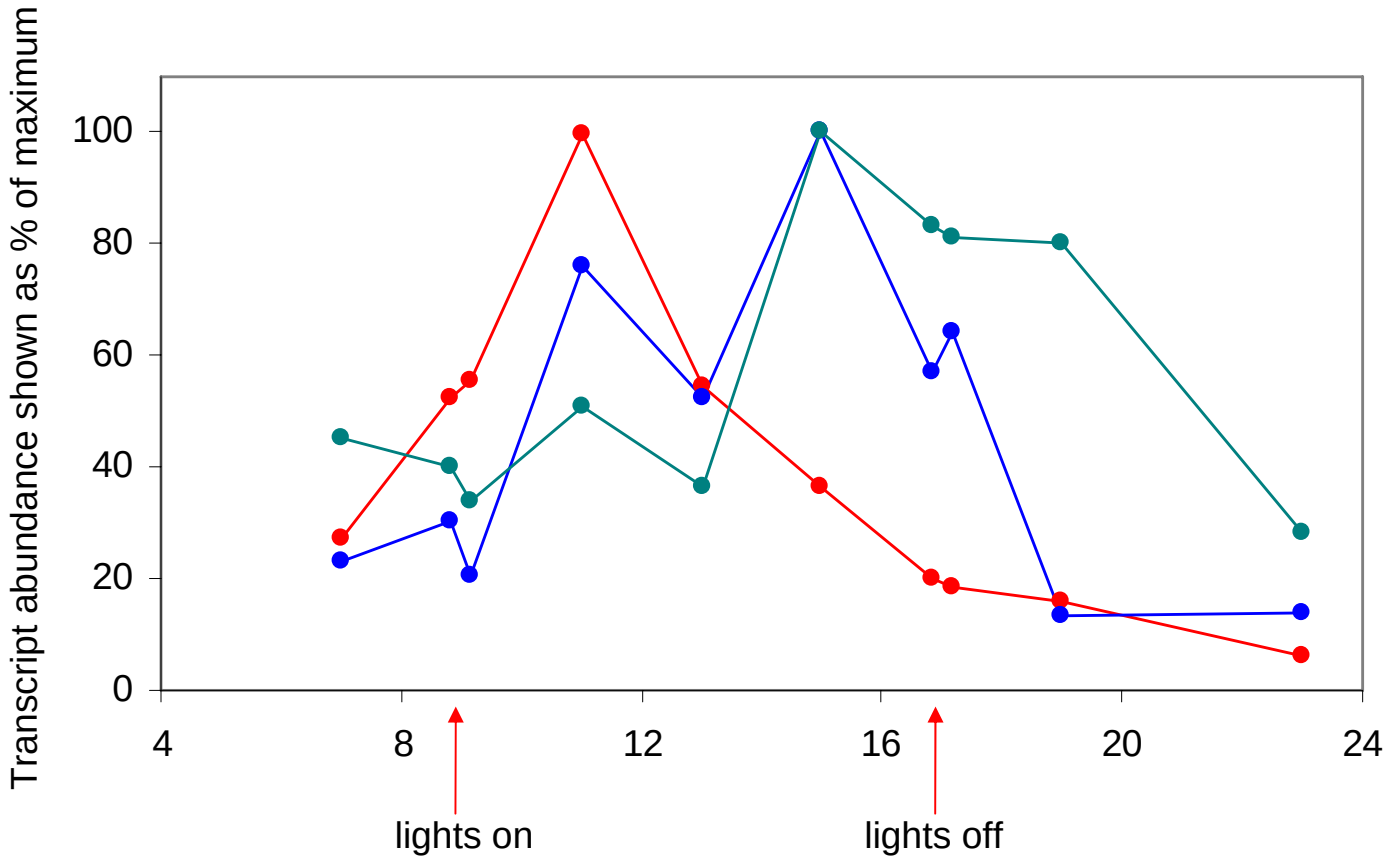


Figure 1. Transcript abundance of *PDX1(2)* (red), *PDX1(5)* (blue), and *PDX2* (green) over a 24 hour cycle. Transcript abundance shown as percent of maximum value.

## APPENDIX C: A THEORY LINKING THE ANTIOXIDANT PROPERTIES OF VITAMIN $B_6$ TO CELLULAR SIGNALING

Organisms tend to be efficient. As organisms increase in complexity, there seems to be a pattern of finding new functions for molecules already present for other uses. For example, nucleotides, the basic building blocks of DNA and RNA, have also found an important function in cell signaling. Similarly, vitamins have multiple functions. Vitamins B, C, and E, while being important cofactors, have also been found to protect cells from oxidative damage. Just recently, vitamin C was proposed to also involved in regulating the redox status of the apoplast, thereby affecting redox-sensitive signaling pathways within the cell (Pignocchi and Foyer, 2003). I think vitamin $B_6$ may also be tied to apoplast redox status and cell signaling, though through a mechanism different from vitamin C.

This theory is based on several pieces of evidence:

$B_6$ vitamers have been shown to be potent quenchers of active oxygen species (Bilski et al., 2000; Jain and Lim, 2001; Kannan and Jain, 2004; Stocker et al., 2003). Ohta and Foote studied the ability of vitamin $B_6$ to quench one form of active oxygen species, singlet oxygen, and found that $B_6$ molecules are rapidly destroyed during the quenching process (2002).

Yeast (both *S. pombe* and *S. cerevisiae*) and *E. coli* efficiently excrete significant amounts of $B_6$ vitamers into the medium (Argoudelis, 1999; Chumnantana et al., 2001;

Hirose et al., 2000; Nakano et al., 1999), an observation that suggests a function other than serving as an enzyme co-factor in amino acid synthesis.

Pyridoxal reductase, a $B_6$ salvage pathway enzyme, is thought to be an external protein associated with the cell membrane (Nakano et al., 1999). The authors proposed that pyridoxal reductase may be involved in pumping $B_6$ out of the cell.

The active form of vitamin $B_6$, pyridoxal 5'-phosphate, is a known antagonist to P2X receptors (Ralevic and Burnstock, 1998). P2X receptors are ligand-gated channels that upon activation by ATP allow $Na^+$ and $Ca^{2+}$ to pass from the apoplast into the cytoplasm (Communi and Boeynaems, 1997; Ralevic and Burnstock, 1998).

A study using rats linked hypertension to diets deficient in vitamin $B_6$ (Dakshinamurti et al., 1998). The hypertension in the vitamin $B_6$-deficient rats was thought to be due to increased intracellular levels of $Ca^{2+}$. As both $Ca^{2+}$ channel blockers and pyridoxal 5'-phosphate inhibited the $Ca^{2+}$ influx and reduced the signs of hypertension, pyridoxal 5'-phosphate may be an endogenous modulator of calcium channels.

Studies on salt stress tolerance in Arabidopsis identified pyridoxal kinase, an enzyme involved in the salvage pathway for vitamin $B_6$, as being hypersensitive to $Na^+$, $K^+$, and $Li^+$ ions (Shi et al., 2002). The authors propose that pyridoxal 5'-phosphate may modulate these ion channels in roots.

Putting this evidence together, my theory follows that pyridoxal 5'-phosphate is pumped out into the intercellular space (or sent to the outside of the vacuole inside the

cell) where it antagonizes different ion channels. When active oxygen species are produced in the apoplast, pyridoxal 5'-phosphate is destroyed (acting as an antioxidant in the process) and therefore no longer antagonizes ion channels. This allows an insurgence of ions, in particular $Ca^{2+}$ and $Na^{+}$, into the cell. This influx of ions, a primary and necessary event during a cell's response to stress, initiates signaling cascades to mount a protective response.

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