ABSTRACT

RUESCHHOFF, ELIZABETH ELLEN. Vitamin B_6 Metabolism in *Arabidopsis thaliana*. (Under the direction of Dr. Margaret E. Daub.)

Vitamin B_6 is an important coenzyme in over one hundred different cellular reactions and processes, including those of amino acid metabolism, heme and chlorophyll biosynthesis, ethylene biosynthesis, fatty acid metabolism, transcriptional regulation and response to oxidative stress. There are six different forms of vitamin B_6 which are termed vitamers and include pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM) and their phosphorylated vitamers, PLP, PNP and PMP respectively. Vitamin B_6 is synthesized de novo by two different enzymatic pathways, the DXP (1'-deoxy-D-xylulose-5'-phosphate)- dependent pathway of *E. coli* and a few other bacteria, and the DXP- independent pathway found in almost all other organisms, except for animals. In addition to the de novo pathways, another pathway is found in all organisms, and functions to convert the six different vitamer forms between each other. This pathway is called the "salvage pathway." Research reported in this dissertation focuses on genes involved in vitamin B_6 biosynthesis in the model plant Arabidopsis.

In the first study, the four Arabidopsis de novo pathway genes (*PDX1.1, PDX1.2, PDX1.3* and *PDX2*) were characterized for expression and response to environmental stress conditions. Under control conditions, *PDX1.1* and *PDX1.3* had the highest expression, with *PDX1.2* the lowest, and *PDX2* intermediate. *PDX1.1*, *PDX1.3*, and *PDX2* were upregulated by high light, chilling, and drought. *PDX1.2*

was upregulated in response to ozone. PDX2 fused to the green fluorescent protein was used to localize the PLP synthase enzyme complex in the nucleus and cellular membranes.

In the second study, two different Arabidopsis mutants of vitamin B_6 metabolism, *pdx1.3* and *sos4,* were characterized. *pdx1.3* is deficient in one of the homologs of the PLP synthase complex, while *sos4* lacks a functional pyridoxine kinase, which phosphorylates the non-phosphorylated vitamers within the salvage pathway. These two mutants were found to have significantly different levels of B6 vitamers. Compared to wild type plants, the *pdx1.3* mutant contains 40% vitamin B_6 of wild type plants, while the *sos4* mutant has 150% vitamin B_6. However, both mutants were shown to share a large number of phenotypes, including chlorosis, decreased plant growth, altered chloroplast ultrastructure, altered expression of carbohydrate metabolism and photosynthetic genes, and severely decreased root growth when grown on exogenous sucrose. Many of the phenotypes could be explained by a deficiency of vitamin B_6 within the chloroplast, and it was hypothesized that chloroplasts of both mutants were deficient in vitamin B_6. Assays of vitamin B_6 content in chloroplasts of the *pdx1.3* and *sos4* mutants showed that as compared to wild type, both mutants had significantly reduced levels of phosphorylated vitamers in chloroplasts with no difference in the levels of non-phosphorylated vitamers. Therefore, even though *sos4* mutants have a surplus of vitamin B_6 in their leaf tissue, they are deficient in vitamin B_6 in their chloroplasts, resulting in phenotypes similar to vitamin B_6- deficient *pdx1.3* mutants. This work

identifies an essential role for pyridoxal kinase in maintenance of vitamin B_6 within the chloroplast.

Another hypothesis that was considered to explain the common phenotypes in the *pdx1.3* and *sos4* mutants was that the PDX1.3 and SOS4 proteins interacted with each other or in a common pathway, and that a mutation in either one of the proteins would produce the same phenotype. This work did not identify an interaction between the PDX1.3 and SOS4 proteins, but did identify a possible novel protein interaction of the PDX1.3 protein with a relatively uncharacterized zinc-binding oxidoreductase (At3g28670). Further work needs to be performed to confirm and characterize this interaction and its possible role in the *pdx1.3* mutant phenotype.

Vitamin B_6 Metabolism in *Arabidopsis thaliana*

by
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DEDICATION

This dissertation is dedicated to all of my students out there who inspired me to continue my own education in the hopes of improving theirs.

BIOGRAPHY

My name is Elizabeth Ellen Rueschhoff, although most people know me as "Beth." I was born in Puerto Rico as the third child of an Air Force family, and I travelled a great deal as a child. I don't claim any particular place as my "home;" I am comfortable wherever I hang my hat. I have lived in many different states, including Georgia, Louisiana, Ohio, Florida, Colorado, Wyoming and North Carolina. I have also lived in several countries other than the United States, including Japan, Jordan, Italy and Holland. I received my Bachelor's degree from the University of Colorado, Colorado Springs (UCCS). Life circumstances brought me to North Carolina, where I fulfilled a life-long desire to become a teacher. After teaching high school Biology for four years, I decided to return to school to obtain my PhD at NC State University, where I had the good fortune of landing in the lab of Dr. Margaret Daub.

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It seems impossible to me to adequately thank and acknowledge all of those who are responsible for helping me to achieve this goal.

I would like to start by thanking Dr. Margo Daub, who was always supportive and put up with my stubbornness at every turn. Also, Dr. Sonia Hererro, you are worth more than your weight in gold. I couldn't have made it without you. Thanks also to all the other members of the lab: Dr. Nafisa Gomaa, Dr. Eddy Vélëz, Dr. Sheri Denslow, Dr. Tanya Taylor, Dr. Eugenia Gonzalez, Dr. Alongkorn Amnuaykanjanasin, Aydin Beseli, Andrew Mealin, and Matt Hargrove. All of you contributed to my success.

I would also like to thank Dr. Heike Sederoff and the members of her lab for accepting me as one of their own. I enjoyed my time with you all and learned so much! Of course, I can't forget the other members of my committee for their guidance and support: Dr. Jose Alonso, Dr. Wendy Boss and Dr. Niki Robertson.

Last, but not least, I must thank my husband, who stuck with me through thick and thin, made sure I had food to eat and clean clothes to wear and was a huge source of support. He listened to me talk about my work, even if he didn't understand a word of what I said, and was always there as a shoulder to lean on when I needed it.

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Chapter 1: Literature Review

Vitamin B_6 – The Molecule

The term vitamin B_6 refers to a collection of related molecules called vitamers. The six vitamers include pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives pyridoxine 5'-phosphate (PNP), pyridoxal 5'phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) (Fig. 1). Vitamin B_6 was initially discovered in the early 1930's when Ohdake, who was studying vitamin B_1 from rice-polishings, first reported the molecular formula for two unknown bases isolated from "active oryzanin." One of these unknown bases was $C_8H_{10}NO_3$ (Ohdake, 1932). However, this formula was later revealed to be incorrect, as noted by Wiardi, who later attributed the compound to what had, by then, been established as vitamin B_6. The corrected formula was published as $C_8H_{12}O_3N.HCl$ (Wiardi, 1938). By the end of the decade, several other groups had also isolated vitamin B_6 (Gyorgy, 1938; Lepkovsky, 1938). In 1939, Gyorgy and Eckardt suggested that vitamin B_6 be referred to as pyridoxine, and the first laboratory synthesis for pyridoxine was reported (Gyorgy and Eckardt, 1939; Harris and Folkers, 1939).

Vitamin B_6 is a water-soluble vitamin that is required for a large number of enzymatic reactions. Over one hundred different enzymes use vitamin B_6 as a

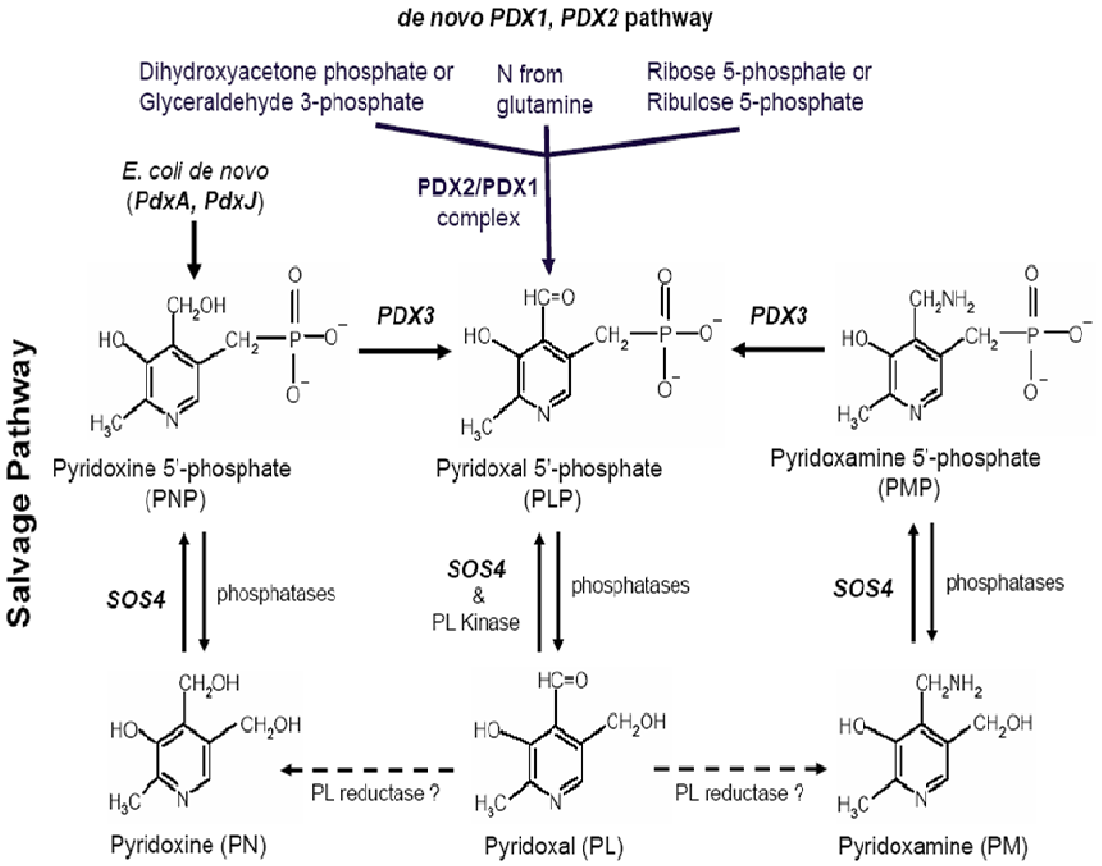

Figure 1. The vitamin B_6 vitamers and their "salvage pathway." Six different vitamin B_6 vitamers are interconverted between each other in the salvage pathway. The six vitamers are pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated vitamers, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). PLP, the active vitamer, is synthesized directly in many organisms by a de novo biosynthetic pathway. It can also be synthesized by conversion of PNP or PMP by PDX3, a pyridoxine (pyridoxamine) 5'-phosphate oxidase. Phosphorylated forms of the vitamers are converted to non-phosphorylated forms by phosphatases. Non-phosphorylated vitamers are phosphorylated by kinases including a PL(PN,PMP) kinase (SOS4) that phosphorylates all three non-phosphorylated vitamers and a PL-specific kinase that phosphorylates PL only. PL is hypothesized to be converted to PN and PM by a PL reductase.

coenzyme to complete their reactions (Mittenhuber, 2001). The active vitamer form is pyridoxal 5'- phosphate (PLP). PLP is required by a variety of different cellular reactions, including reactions involved in amino acid metabolism (Drewke and Leistner, 2001), fatty acid metabolism (Horrobin, 1993; Nakamura and Nara, 2004), heme and chlorophyll biosynthesis (Tsang et al., 2003), auxin biosynthesis (Tao et al., 2008), ethylene synthesis (Capitani et al., 1999), and carbohydrate metabolism (Shimomura et al., 1980; Takagi et al., 1981; Fukui et al., 1982; Preiss et al., 1991). PLP has also been shown to play a role in transcriptional regulation of steroid hormone receptors, to interact with the nuclear transcriptional regulator RIP140 in mice, and is also likely to act as an antioxidant in the presence of biotic and abiotic stresses (Tully et al., 1994; Ehrenshaft et al., 1999; Denslow et al., 2005; Huq et al., 2007). Because vitamin B_6 functions in so many diverse roles, it is important to understand the synthesis and function of this important coenzyme.

Functions of vitamin B_6

Vitamin B_6 and amino acid metabolism

Vitamin B_6 is perhaps most well known for its role as a coenzyme in amino acid metabolism, including decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions (Wiesinger and Hinz, 1984; Drewke and Leistner, 2001). In these reactions, vitamin B_6 forms a Schiff base construct with an amino

acid in the enzyme, most commonly associating with leucine residues (Fig. 2). In this reaction, PLP essentially acts as an electron sink, which destabilizes the bonds around the chiral center of the amino acid undergoing enzymatic conversion. This causes three remaining bonds around the chiral carbon of the amino acid to be weakened, facilitating the decarboxylation, transamination, and deamination reactions.

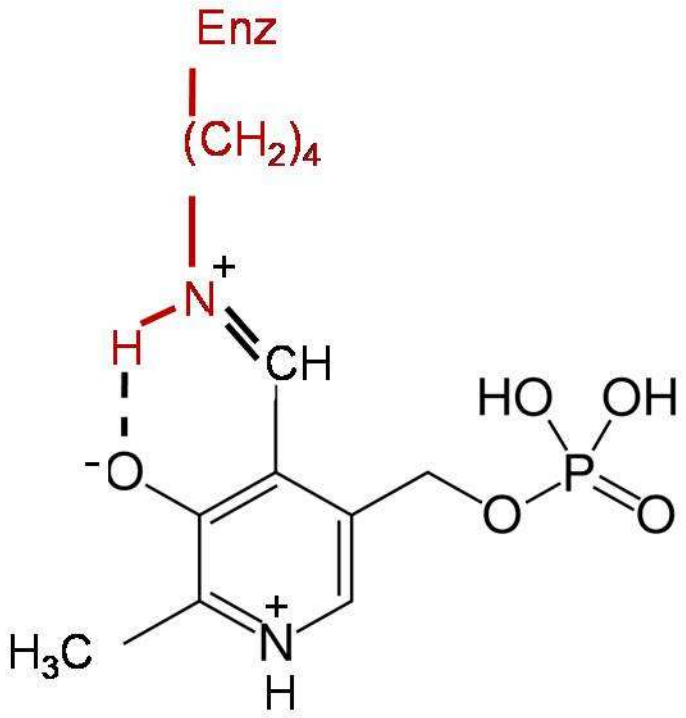

Figure 2. Schiff base construct between the coenzyme PLP and a lysine of the enzyme molecule. PLP is shown in black and the lysine residue of the enzyme is shown in red. "ENZ" represents the remainder of the enzyme molecule.

Vitamin B_6 and fatty acid metabolism

Deficiency of vitamin B_6 has been shown to result in a decrease in activity of delta-6-desaturase (Bordoni et al., 1998). Delta-6-desaturase is the rate limiting step in the formation of γ-linoleic acid, which is an essential fatty acid in cellular metabolism (Horrobin, 1993). While both of these studies were performed in animals, γ-linoleic acid is found in high abundance in vegetable oils and is thought to play a role in inflammatory disease and is used as a precursor for prostaglandins. Prostaglandins are required for proper functioning of the immune system.

Vitamin B_6 and ethylene biosynthesis

Vitamin B_6 is a required cofactor for the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosyl –L-methionine (SAM) by the enzyme ACC synthase (ACS) (Capitani et al., 1999). This enzyme is the rate-limiting step in ethylene biosynthesis, and therefore is a key point of regulation of synthesis of this essential plant hormone (Capitani et al., 1999). Ethylene plays a variety of roles in plant development including seed germination, growth of both the root and the shoot, development of flowers, ripening of fruit, leaf and flower abscission, and response to both biotic and abiotic stress, including flooding, chilling, and dehydration (Capitani et al., 1999).

Vitamin B_6 and auxin biosynthesis

In plants, indole-3-acetic acid (IAA), the key auxin in most plants, is synthesized through two separate pathways, the tryptophan (Trp)-dependent pathway or the Trp-independent pathway (Woodward and Bartel, 2005; Cheng et al., 2006). Vitamin B_6 is required for IAA synthesis in the Trp-dependent pathway, where vitamin B_6 has been shown to be required for function of SAV3, an aminotransferase which catalyzes the formation of indole-3-pyruvic acid (IPA) from L-tryptophan (L-Trp) (Tao et al., 2008). Indole-3-pyruvic acid is an intermediate in IAA biosynthesis (Truelsen, 1972). IAA acts as a signaling molecule in plant development, including processes such as patterning in the root and in the shoot, determination of apical dominance, tropic responses and embryogenesis (Muto et al., 2007).

Vitamin B_6 and heme and chlorophyll synthesis

Vitamin B_6 is required for two different reactions in the pathway which leads to heme and to chlorophyll biosynthesis (Vavilin and Vermaas, 2002). The first reaction is the ligation of glutamate to $tRNA_{Glu}$ by glutamyl-tRNA synthetase (GTS). PLP is also required for the function of glutamate-1-semi aldehyde aminotransferase (GSA), in which aminolevulinic acid is formed by the transamination of glutamate-1-semialdehyde to form aminolevulinic acid (ALA), which undergoes further modifications, ultimately resulting in the formation of both heme and chlorophyll.

The formation of aminolevulinic acid is the rate limiting step in chlorophyll biosynthesis (Vavilin and Vermaas, 2002).

Vitamin B_6 and carbohydrate metabolism

PLP has been shown to serve as a coenzyme for both starch and glycogen phosphorylases. Starch and glycogen phosphorylases are responsible for the removal of a glucose molecule from the non-reducing ends of glycogen and starch (Shimomura et al., 1980; Takagi et al., 1981; Withers et al., 1981; Fukui et al., 1982; Palm et al., 1990). Furthermore, PLP has been shown to inhibit ADP-glucose pyrophosphorylase, a key chloroplast enzyme involved in starch biosynthesis. PLP has also been shown to inhibit the export of triose phosphates by the triose phosphate transporter (TPT) (Fliege et al., 1978; Preiss et al., 1991), a protein that regulates the balance of CO_2 partitioning between starch synthesis in the chloroplast and sucrose synthesis and glycolysis in the cytoplasm.

Vitamin B_6 and steroid function

PLP has been implicated in the activation of steroid receptors (Tully et al., 1994). PLP has been shown to bind to human glucocorticoid receptors in vitro, and to change their sedimentation coefficient from 8S (inactive form) to 4S (active form) (O'Brien and Cidlowski, 1981). Only incubation with PLP had this effect on the

sedimentation coefficient, and other vitamers such as PN and PMP had no effect on the sedimentation rates. This suggests that only PLP interacts with the receptor protein. The change in conformation also occurs with other types of hormone receptors, including progesterone and estrogen receptors (Nishigori and Toft, 1979; Muldoon and Cidlowski, 1980). Not only has PLP been shown to bind to hormone receptors, but it has also been shown that changes in PLP levels within the cell result in modulated expression of glucocorticoid-induced gene expression (Tully et al., 1994).

Vitamin B_6 and antioxidant activity

Ehrenshaft et al. demonstrated that vitamin B_6 is able to quench singlet oxygen via chemical quenching at rates similar to that of vitamins C and E (Ehrenshaft et al., 1999). Subsequently, Denslow et al. (2005) showed that B_6 vitamers quench superoxide and have antioxidant activity. This same study demonstrated that tobacco B_6 biosynthetic genes were down-regulated during a bacterially-induced hypersensitive reaction (HR), a defense response that requires active oxygen. Further, co-infiltration of tobacco leaves with bacteria and pyridoxine significantly delayed the on-set of the HR caused by an avirulent bacterial pathogen and enhanced disease caused by a virulent bacterial pathogen. These results were consistent with B_6 vitamers acting as antioxidants in vitro.

Many other studies have linked B_6 vitamers with antioxidant activity. B_6 vitamers have been shown to prevent lipid peroxidation and protein oxidation in blood due to high levels of glucose and H_2O_2. In addition, vitamin B_6 has been shown to protect against superoxide-mediated eye damage in diabetics (Jain and Lim, 2001; Jain et al., 2002; Kannan and Jain, 2004). In rats, a deficiency of vitamin B_6 caused an impairment of the oxidative stress response system, which in turn resulted in an increased in oxidative stress in the liver tissue (Seyithan, 2005). Paraquat treatment increased expression of *PDX1* in *B. subtilis* (Antelmann et al., 1997), and hydrogen peroxide treatment of *S. pombe* increased transcript abundance of both *PDX2* and the gene encoding pyridoxal reductase (Chen and Xiong, 2005). A yeast mutant for the PNP(PMP) oxidase were shown to be sensitive to H_2O_2 (Sang et al., 2007). In *A. thaliana*, pyridoxine was shown to decrease $^1O_2$-induced death in *flu* mutants (Danon et al., 2005), and a decrease in vitamin B_6 content was shown to result in an increase of lipid peroxidation (Chen and Xiong, 2005).

Vitamin B_6 and maintenance of DNA integrity

In 2007, Kanellis et al. identified a novel role for vitamin B_6 within the cell in *S. cerevisiae* (Kanellis et al., 2007). They showed that vitamin B_6 was required for proper maintenance of DNA integrity, and that mutation of BUD16, pyridoxal kinase, causes an increase in gross chromosomal rearrangements at multiple loci of the yeast

genome. In addition, yeast deficient in PLP were found to have a higher incidence of uracil incorporation in their nuclear DNA, which could cause a degradation of genome integrity.

De novo pathways of vitamin B_6 metabolism

Most organisms synthesize vitamin B_6 de novo, including bacteria, protists, fungi and plants. In general, animals cannot make vitamin B_6 and must therefore obtain this nutrient from their diet. Foods rich in vitamin B_6 include turkey, chicken, fish, eggs, bananas, nuts, fortified cereals and green, leafy vegetables. Only one instance of animals having the genes to make their own vitamin B_6 has been reported. Recently, a study was published showing that *Heterodera glycines*, the soybean cyst nematode, contains homologues to the vitamin B_6 de novo pathway enzymes SNZ and SNO (Craig et al., 2008). These homologues contain nematode-like introns, polyadenylated mRNAs and map to the soybean cyst nematode (SCN) genetic linkage map, which suggests that these genes are part of the nematode genome. However, homology and phylogenetic analyses suggest that the genes originate from prokaryotes, indicating that these genes were acquired by the nematode through horizontal gene transfer. This appears to be the first report of the vitamin B_6 de novo pathway in an animal.

Two different de novo biosynthetic pathways exist in nature (Figure 2). The first pathway is referred to as the "DXP-dependent pathway." This pathway is found in *Escherichia coli* and a handful of other prokaryotes. Until 1999, this was the only de novo pathway described in nature. The second pathway is referred to as the "DXP-independent pathway", and is the predominant pathway found in nature. This pathway was discovered in 1999 during the investigation of genes involved in autoresistance of *Cercospora* fungi to their own toxin cercosporin (Ehrenshaft et al., 1999). It is by this pathway that most organisms synthesize PLP, the active form of vitamin B_6. While both pathways synthesize a vitamin B_6 vitamer, they differ from each other in substrate, product and in their enzymatic reactions. The two pathways are summarized in Figure 3 and are reviewed below.

"DXP dependent" de novo synthetic pathway

The DXP-dependent pathway, found in *E. coli* and a handful of other bacteria, was the first vitamin B_6 synthetic pathway identified (Figure 2) (Hill et al., 1996; Drewke and Leistner, 2001). In this pathway, nitrogen from glutamate is combined with 3-hydroxy-4-phospho-hydroxy-α-ketobutyrate to form 4-phospho-hydroxy-threonine by the SerC protein. This intermediate is converted to 3-hydroxy-1-amino-acetone-3-phosphate by the PdxA protein. In turn, 3-hydroxy-1-amino-acetone-3-

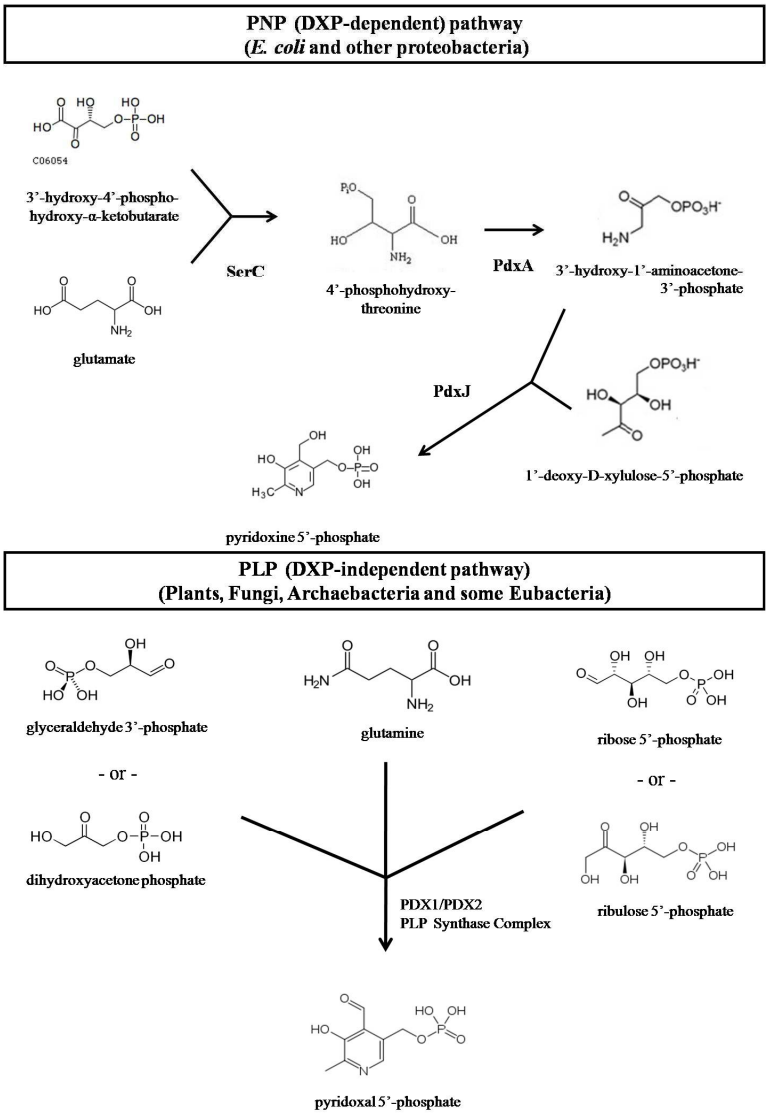

Figure 3. PNP and PLP de novo biosynthetic pathways. PNP pathway (DXP-dependent) was the first pathway described and is found in *E. coli* and other proteobacteria. The pathway involves three enzymes, SerC, PdxA, and PdxJ using substrates as shown and results in the formation of pyridoxine 5'-phosphate, which must be converted to pyridoxal 5'-phosphate by the salvage pathway. The PLP (DXP-independent) pathway is found in plants, fungi, archaebacteria and some eubacteria. In this pathway, pyridoxal 5'-phosphate is synthesized by directly by a complex of two proteins, a glutaminase (PDX2) and a ring closure protein (PDX1). Pathways differ in substrates, nitrogen source, enzymes, and in the product.

phosphate is combined with 1-deoxy-D-xylulose-5'-phosphate by the PdxJ protein to form pyridoxine 5'-phosphate. It is noteworthy that the product of this pathway is PNP, and not PLP. The organisms that synthesize vitamin B_6 by this pathway must convert their PNP to PLP before it can be used in cellular processes. Because the product of this pathway is PNP, the DXP-dependent pathway may be seen as less efficient than the second pathway, which is referred to as the DXP-independent pathway.

"DXP-independent" de novo synthetic pathway

In 1999, Ehrenshaft and co-workers showed that the SOR1 (singlet oxygen resistance) gene was essential for vitamin B_6 synthesis in the fungi *Cercospora nicotianae* and *Aspergillus flavus* (Ehrenshaft et al., 1999). This gene, although highly conserved in nature, was not homologous to any of the genes known to encode proteins involved in vitamin B_6 synthesis in *E. coli*. This suggested that there were two pathways of vitamin B_6 found in nature, and identification of this gene was the first step in characterizing the DXP-independent de novo pathway, which is now known to be the predominant pathway found in nature. In the ten years since this discovery, genes encoding proteins in the DXP-independent pathway have been identified and characterized from many organisms (see Table 1). The most well-characterized genes are found in *Saccharomyces cerevisiae* (*SNO1* and *SNZ1)* and *Arabidopsis thaliana* (*PDX1* and *PDX2*) (Dong et al., 2004; Denslow et al., 2005;

Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Denslow et al., 2007; Gonzalez et al., 2007; Tambasco-Studart et al., 2007; Chen and Xiong, 2009). In this pathway, the PDX1 and PDX2 proteins form a PLP synthase complex, where it has been shown that the proteins interact with each other and work together in a protein complex to synthesize PLP (Fig. 2). The direct formation of PLP eliminates the need for the pathway end product to undergo any further modifications in order to be used in the cell.

Details on the characterization of the pathway in different organisms are explained below, but all share the following general characteristics (Bauer et al., 2004; Dong et al., 2004; Raschle et al., 2005; Flicker et al., 2007; Tambasco-Studart et al., 2007; Raschle et al., 2009). The DXP-independent pathway utilizes glutamine as the nitrogen source instead of glutamate, the nitrogen source used in the DXP-dependent pathway of *E. coli*. Instead of having multiple steps, synthesis of PLP is accomplished in one step by a PLP synthase protein complex made up of two enzymes, one which functions as a glutaminase and the other as a ring closure protein. In this reaction, glutamine is combined with a three carbon compound (either glycaraldehyde 3-phosphate or dihydroxyacetone phosphate) and a five carbon sugar (either ribose 5'-phosphate or ribulose 5'-phosphate). The raw materials of this pathway not only intimately connect vitamin B_6 and amino acid metabolism through

glutamine, but also tie vitamin B_6 to the energy pathways of the cell through the triose/pentose phosphate metabolite pools.

Vitamin B_6 synthesis has been characterized in a number of different organisms. Among these are *C. nicotianae, Nicotiana tabacum, Aspergillus nidulans, S. cerevisiae*, *Bacillus subtilis, Plasmodium falciparum, Thermotoga maritima,* and *A. thaliana.* Descriptions of what is known in each system are outlined below, and the proteins are summarized in Table 1.

Table 1. Summary of Proteins Identified in DXP-Independent Vitamin B_6 Synthetic Pathways

Organism	Ring Closure Protein	Glutaminase
Bacillus subtilis	YaaD	YaaE
Saccharomyces cerevisiae	SNZ1 SNZ2 SNZ3	SNO1 SNO2 SNO3
Plasmodium falciparum	PDX1	PDX2
Cercospora nicotianae	PDX1	PDX2
Thermotoga maritima	YaaD	YaaE
Arabidopsis thaliana	PDX1.1 PDX1.2 PDX1.3	PDX2
Nicotianae tabacum	PDX1 (two copies)	PDX2
Aspergillus nidulans	pyroA	pyroB

Cercospora nicotianae

The vitamin B_6 DXP-independent de novo pathway was first discovered in *C. nicotiana.* Ehrenshaft et al. first reported the discovery of what was thought to be a

novel gene involved in vitamin B_6 metabolism while studying *C. nicotianae*'s resistance to its own toxin (Ehrenshaft et al., 1999). Originally referred to as *SOR1,* identification of *PDX1* was the first indication that there are two different biosynthetic pathways found in nature. This study was followed by another in 2001, in which *PDX2* was identified in *C. nicotianae* as the second gene involved in pyridoxine biosynthesis (Ehrenshaft and Daub, 2001). Searches of sequence databases showed that homologues to *PDX1* and *PDX2* were found in organisms in diverse kingdoms including fungi, plants, archaebacteria, protists, and many eubacteria. Significantly, homologues were not found in *E. coli* and other proteobacteria that contain *PdxA* and *PdxJ*, suggesting the presence of two distinct pathways in nature. The diverse number of organisms with proteins homologous to PDX1 and PDX2 suggested that the *PDX1/PDX2* pathway is the predominant pathway found in nature. This conclusion was supported by a comparative genomics study by Mittenhuber (Mittenhuber, 2001). In addition to identifying the genes involved in the DXP-independent de novo pathway, vitamin B_6 was revealed to be a potent quencher of singlet oxygen, with quenching constants similar to vitamins C and E (Ehrenshaft et al., 1999). This was the first indication that vitamin B_6 may play a role in antioxidant defenses in cells.

N. tabacum

Denslow et al. (2005) isolated and characterized the *PDX1* and *PDX2* homologues from tobacco, as part of a study to determine the role for vitamin B_6 in plant defense responses. Amplification of the genes using a degenerate primer strategy reproducibly recovered two sequences for *PDX1* that differed by 15 nucleotides but resulted in identical amino acid sequences. These may be two alleles or two copies, as tobacco is an allotetraploid. Southern and sequence analysis suggested the presence of only one copy of *PDX2*. The PDX1 sequences were highly conserved, showing 80-90% amino acid homology with plant homologues in sequence databases. PDX2 was less conserved. Gene expression studies demonstrated that *PDX1* is more highly expressed than *PDX2*. Both genes were down-regulated during the induction of the hypersensitive defense response, consistent with a role for vitamin B_6 as an antioxidant.

Herrero and Daub (2007) attempted to increase vitamin B_6 production in tobacco. In these experiments, they expressed PLP synthase genes from *C. nicotianae* in *N. tabacum*. However, only one line was recovered with significantly increased levels of B_6 vitamers, and the increase was small (about 17% higher than wild type or a vector control transformed line). In transformants expressing the *PDX1* and *PDX2* transgenes, expression of endogenous tobacco genes was decreased,

suggesting that the de novo pathway is under tight regulation (Herrero and Daub, 2007).

A. nidulans

Shortly after Ehrenshaft et al. identified genes involved in vitamin B_6 metabolism in *C. nicotianae*, Osmani et al. reported identification of the *pyroA* gene in *A. nidulans* (Osmani et al., 1999) as a homologue of PDX1, one of the two components of the PLP synthase complex. This highly conserved gene encodes a protein 304 amino acids in length with a predicted MW of 32.4 kDa. In their study, *A. nidulans* strains mutated in *pyroA,* a *PDX1* homolog, and *pyroB,* a *PDX2* homolog were sensitive to the photosensitizer methylene blue. This sensitivity of the *pyroA* mutant was able to be rescued by transformation with a functional *pyroA* gene. In addition, supplementation of both *pyroA* and *pyroB* mutants with exogenous pyridoxine when grown in the presence of methylene blue reversed methylene blue sensitivity, indicating that vitamin B_6 plays an important role in this organism's response to oxidative stress.

S. cerevisiae

The two vitamin B_6 biosynthetic proteins identified in *S. cerevisiae* are known as SNO and SNZ, which are homologous to PDX2 and PDX1, respectively. There are

three different homologs of both proteins, referred to as SNZ1, SNZ2 and SNZ3, and SNO1, SNO2 and SNO3. SNZ1 and SNO1 are located on chromosome XIII, SNZ2 and SNO2 are located on XIV and SNZ3 and SNO3 are located on chromosome VI (Rodriguez-Navarro et al., 2002). The *SNZ* genes were originally identified and characterized as novel genes that were upregulated during stationary phase (Braun et al., 1996); the *SNO* genes were described as being in close proximity to and co-regulated with the SNZ genes (Padilla and Fuge, 1998). After the discovery of the *C. nicotianae* pathway, both SNZ1 and SNO1 were shown to be required for growth in the absence of vitamin B_6 (Rodriguez-Navarro et al., 2002). Single mutants in these proteins showed differential reductions in growth in media lacking vitamin B_6. While single mutants of SNO1 and SNZ1 were unable to grow without vitamin B_6, single mutants of SNZ2, SNZ3, SNO2 and SNO3 were not compromised in growth when grown in the absence of vitamin B_6, indicating that SNZ1 and SNO1 are the dominant homologs in vitamin B_6 metabolism. In addition, the SNZ triple mutants and SNO triple mutants also required the addition of vitamin B_6 for proper growth (Rodriguez-Navarro et al., 2002). In 2004, Dong et al. expressed the SNZ1 and SNZ2 proteins in *E. coli*, and demonstrated that the two proteins formed a complex that had glutaminase activity (Dong et al., 2004).

B. subtilis

In 2002, Sakai et al. reported that YaaD and YaaE were involved in vitamin B_6 synthesis in *B. subtilis* (Sakai et al., 2002). *B. subtilis* has only one copy of each gene. These genes are expressed most highly during middle- to late-exponential phase, and were expressed least during stationary phase (Sakai et al., 2002). Much of the pioneering work characterizing the PLP synthase complex has been done in *B. subtilis*. Bauer, et al. published the crystal structure of YaaE in 2004, and, through protein modeling, proposed that YaaD interacts with YaaE to form an enzyme complex (Bauer et al., 2004). Raschle, et al. confirmed the glutaminase activity of the YaaD and YaaE enzyme complex, and showed that glutaminase activity only occurs in the presence of both enzyme subunits (Raschle et al., 2005). They also showed that the substrates include ribulose 5'-phosphate, and that either glyceraldehyde 3'-phosphate or dihydroxyacetone phosphate participated in the synthesis of PLP. These findings were also independently reported by Burns, et al., who showed that ribose 5'-phosphate could also serve as a substrate (Burns et al., 2005). Burns, et al. were the first group to report the reconstitution of the synthesis of PLP from glutamine, ribose 5'-phosphate and glyceraldehyde 3'-phosphate. In addition, they also identified three partial reactions of the complex: pentose isomerization, triose isomerization and imine formation (Burns et al., 2005).

P. falciparum

Vitamin B_6 synthesis in this malarial parasite was described by Wrenger, et al. in 2005. They reported that vitamin B_6 is synthesized by a complex similar to the other characterized DXP-independent pathway complexes (Wrenger et al., 2005). In *P. falciparum,* PfPdx1 and PfPdx2 interact to form a protein complex with glutaminase activity. Neither subunit is active unless bound to each other (Gengenbacher et al., 2006). The binding site of L-glutamine is located at the interface between the PDX1 and PDX2 subunits (Flicker et al., 2007). The interaction of two subunits of the complex is strengthened almost thirty-fold in the presence of glutamine when compared to those not in the presence of glutamine (Flicker et al., 2007).

Wrenger, et al. also reported that both *PfPdx1* and *PfPdx2* were upregulated in the presence of methylene blue, indicating that the genes of this pathway are involved in responses to singlet oxygen (Wrenger et al., 2005). Northern blotting revealed that expression of these two genes and of the pyridoxal kinase-encoding *PdxK* gene were coordinated in the trophozoite stage of development, which is the stage when the parasite is feeding and growing (Wrenger et al., 2005).

T. maritime

As in *B. subtilis,* the two components of the PLP synthase complex in *T. maritime* are called YaaD and YaaE. Zein et al published the quaternary structure of the PLP synthase complex from *T. maritima* in 2006 (Zein et al., 2006). In this structure, a core is formed by twelve interacting YaaD monomers. Twelve YaaE monomers interact with the twelve YaaD monomers of the core. However, the YaaE monomers do not interact with each other. In this structure, YaaD catalyzes the condensation of ribulose 5'-phosphate, glyceraldehyde 3'-phosphate, and ammonia, and YaaE functions as the glutaminase to catalyze the production of ammonia from glutamine. Ammonia is channeled from the active site of YaaE to the active site of YaaD to complete formation of the pyridoxal 5'-phosphate molecule (Zein et al., 2006).

A. thaliana

The de novo synthetic pathway of *A. thaliana* has been characterized by several different groups, including ours (Chen and Xiong, 2005; Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Denslow et al., 2007; Tambasco-Studart et al., 2007). In *A. thaliana*, the two de novo pathway proteins are called PDX1 and PDX2. There is only one copy of the *PDX2* gene in *A. thaliana*, located on chromosome five (At5g60540). There are three *PDX1* homologs, which have

been termed *PDX1.1*, *PDX1.2* and *PDX1.3*. *PDX1.1* (At2g38230) is located on chromosome 2, *PDX1.2* (At3g16050) is located on chromosome 3 and *PDX1.3* (At5g01410) is located on chromosome 5. PDX1.1 and PDX1.3 are 89% identical to each other. PDX1.2 is more divergent and is only 60% identical to the other two copies (Denslow et al., 2005; Tambasco-Studart et al., 2005; Denslow et al., 2007). A smaller, incomplete copy (PDX1.4) 100% identical to the first 33% of PDX1.1 is located just upstream from the full gene sequence. This short copy is not thought to function in cellular metabolism. None of the *PDX1* homologs have introns, which could suggest prokaryotic origin. It has also been reported that genes required for response to environmental stress contain fewer introns (Jeffares et al., 2008). This supports the role of vitamin B_6 as an antioxidant, important in responding to environmental oxidative stresses. Transcripts of *PDX1.1, PDX1.2* and *PDX1.3* are 1053, 1380 and 1297 bp in length, respectively.

The single copy of PDX2 in *A. thaliana* is required for plant growth and development. The gene is 2180 nucleotides in length and has seven exons, producing a transcript 768 base pairs in length. The protein produced from the transcript is 255 amino acids in length with a predicted molecular weight of 27303 Daltons (Tambasco-Studart et al., 2005). Mutations in this protein have been shown to be embryo-lethal, with embryo development aborting at the heart stage (Tambasco-Studart et al., 2005; Tambasco-Studart et al., 2007). Even though this mutation is

embryo-lethal, one *pdx2* mutant has been recovered (Rueschhoff, unpublished data). Heterozygous plants were supplemented with vitamin B_6 through seed set. Seeds were collected and germinated on artificial growth medium (MS) supplemented with pyridoxine. Germinated seeds were transferred to soil and supplemented with exogenous pyridoxine. Seeds were screened for homozygosity of the insertion. Out of 200 germinated seedlings, only one plant homozygous for the T-DNA insertion mutant was able to be rescued (Rueschhoff, unpublished data). It is unclear why only one mutant was able to be recovered. Because this mutant was very difficult to obtain, no further investigation into this mutation has been done.

Because of the difficulty in obtaining homozygous mutants of PDX2, most of the work on this pathway has concentrated on mutants of the three PDX1 homologs (Chen and Xiong, 2005; Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Denslow et al., 2007; Gonzalez et al., 2007). Of these three homologs, PDX1.3 is most well-characterized. Transcript abundance is highest in this homolog, and *pdx1.3* mutants have displayed the greatest phenotypic changes and alterations in B_6 levels (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006). Further, this gene has been recovered in several screening studies of genes and proteins not thought to be tied to B_6 metabolism (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006).

The earliest studies on *A. thaliana* PDX1 were published in 2005, when Chen and Xiong reported isolating what is now called PDX1.3 from a Yeast-2 Hybrid (Y2H) assay designed to isolate interacting partners of a scaffolding protein involved in responses to drought and salt stress (Chen and Xiong, 2005). The transcript of *PDX1.3* was found to be present in roots, stems, leaves, flowers, and siliques. Expression studies using GUS-driven promoters indicated that this gene is expressed most strongly in vascular tissues and guard cells. In addition, GUS staining was seen in root hairs, mature pollen, filaments, in the tips of young siliques, and at the joint at which the silique meets the pedicel. GUS staining was also prevalent at wounding sites, indicating that PDX1 may be involved in wound response. GUS staining was not inducible by jasmonic acid, ethephon or UV light, and transcripts of the gene did not accumulate when exposed to ABA or salt stress, even though the gene was recovered in a screen for drought and salt stress. Chen and Xiong showed through GFP fusion studies that the protein is localized to the nucleus and in membranes including the plasma membrane. Mutants for the gene were deficient in chlorophyll and root growth when compared to WT plants. Root growth impairment was shown to be due to reductions in both cell elongation and cell division. Roots of seedlings of *pdx1.3* mutants were sensitive to both salt and mannitol, indicating that the PDX1.3 protein is involved in organismal response to both osmotic and ionic stresses. In a subsequent study the same authors showed that the short root phenotype is due to

locally produced signals in the root (Chen and Xiong, 2009). They also showed that *pdx1.3* mutants are deficient in tryptophan dependent auxin biosynthesis in the root, which may explain the short root phenotype.

Independently in 2005, Tambasco-Studart et al. reported the complete de novo pathway of vitamin B_6 synthesis in *A. thaliana*, which was the first report of the pathway in plants (Tambasco-Studart et al., 2005). In this study, they identified the three *PDX1* homologs and named them *At*PDX1.1, *At*PDX1.2 and *At*PDX1.3. Enzymatic assays reported in this study indicated that only *At*PDX1.1 and *At*PDX1.3 are functional in vitamin B_6 synthesis. They also identified the one gene encoding the PDX2 protein. Utilizing transient expression in onion skin peels and *A. thaliana* mesophyll protoplasts, Tambasco-Studart, et al. localized the three PDX1 homologs and PDX2 to the cytoplasm. This result is in contrast to Chen and Xiong, who, as noted above, reported that the PDX1.3 protein was located in membranes and in the nucleus (Chen and Xiong, 2005). De novo synthesis in *A. thaliana* was shown to be independent of deoxyxylulose 5-phosphate and 4-phosphohydroxy-L-threonine (DXP) which were the known substrates for synthesis of vitamin B_6 in *E. coli.* In addition, the authors reconstituted vitamin B_6 synthesis with the *A. thaliana* proteins, and confirmed reports by Burns et al. (Burns et al., 2005) that in the DXP-independent pathway, vitamin B_6 is synthesized from either ribose 5'-phosphate or

ribulose 5'-phosphate, glyceraldehyde 3'-phosphate or dihydroxyacetone phosphate and glutamine.

In 2006, Wagner et al. recovered an EMS mutant of the *PDX1.3* gene (Wagner et al., 2006) from a population of EMS-mutagenized seeds screened for reduced sugar response. The mutant was named *rsr4-1*. This mutant exhibited reduced root growth and smaller, chlorotic leaves. Mapping and sequencing of the mutant gene showed it to be *PDX1.3*, and the authors showed that mutant phenotypes were able to be rescued with addition of exogenous PL to the growth media. Addition of PN, PM or PLP was able to partially restore root growth, although to a lesser extent then supplementation with PL.

Through Y2H analysis and GST pull-down assays (a form of affinity purification), Wagner et al., also showed that each of the PDX1 homologs binds with itself and with the other homologs. In addition, PDX1.1 and PDX1.3 were shown to interact with PDX2. It is noteworthy that PDX1.2 did not interact with PDX2. The mutation in the *rsr4-1* protein disabled the ability of PDX1.3 to interact with the other proteins in the de novo pathway, thereby compromising vitamin B_6 metabolism within the cell (Wagner et al., 2006).

In order to determine whether or not PDX1.1 and PDX1.3 were functionally redundant, Wagner et al. targeted the *PDX1.1* gene through gene silencing technology. Similar to the *rsr4-1/pdx1.3* mutant plants, plants decreased in *PDX1.1*

transcript were chlorotic and had decreased root growth in comparison to WT plants. This abnormal phenotype was attributed to a decrease of the PDX1.1 transcript, since the PDX1.3 transcript was only slightly reduced (Wagner et al., 2006). Silencing of both genes produced plants with leaves that were chlorotic and distorted. Many of the plants died, but those that survived had small flowers that failed to produce seeds. These data suggest that PDX1.1 and PDX1.3 are functionally redundant in vitamin B_6 synthesis.

A subsequent study by Titiz, et al. characterized T-DNA insertion mutants of both *PDX1.1* and *PDX1.3*. While *pdx1.3* mutants had very similar phenotypes when compared to the Wagner mutants, the *pdx1.1* mutant was not decreased in vitamin B_6 content and did not exhibit the chlorotic phenotype of the *pdx1.3* mutant (Titiz et al., 2006). Titiz et al. also attempted to make *pdx1.1/pdx1.3* double mutants (Titiz et al., 2006). They were unable to obtain any double mutants, but were able to isolate some plants that were homozygous for one mutation and heterozygous for the other. These mutants were extremely chlorotic, and the developmental defects seen in the *pdx1.3* mutants were exacerbated. The exacerbated phenotypes combined with the lethality of the double mutants indicate that these genes are at least partially redundant in function. However, there are differences in expression of the genes both in location and in stage of development (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al.,

2006), which indicates that the genes may also have independent functions, requiring maintenance of both genes within the *A. thaliana* genome.

Denslow et al. (2007) characterized the regulation of the *A. thaliana* genes in response to abiotic stress conditions. They showed that under normal conditions, *PDX1.1* and *PDX1.3* were expressed at relatively high levels, with little or no expression of *PDX1.2*. *PDX2* was expressed at intermediate levels. Previous studies by this group (Denslow et al. 2005) showed that the pathway played no role in defense to biotic stress in tobacco, but that the vitamers had antioxidant and active oxygen quenching activity. Denslow, et al. (2007) showed that all three Arabidopsis *PDX1* genes as well as *PDX2* were upregulated in response to high light, chilling, drought, UV, paraquat and ozone. Interestingly, expression patterns of *PDX1.2* were disparate from those of the other genes.

Results from the studies described above have suggested that the PDX1.2 protein does not play a dominant role in the pathway. Titiz et al. investigated *pdx1.2* insertion mutants and found no phenotypic differences when compared to WT plants (Titiz et al., 2006). In addition, the expression level of this gene is very low (Denslow et al., 2007). As previously mentioned, Tambasco-Studart et al., were unable to detect any activity of PDX1.2 in an enzymatic assay (Tambasco-Studart et al., 2005), and PDX1.2 was not shown to interact with PDX2 (Wagner et al., 2006). Lack of transcript and enzyme activity as well as lack of interaction with the

glutaminase subunit of the PLP synthase has led to speculation that the PDX1.2 homolog may not be functional within the cell. However, PDX1.2 protein and transcript have been identified in plants (Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Denslow et al., 2007), and Denslow et al. (2007) showed that transcripts of this gene specifically accumulate with exposure to UV and oxidative stressors such as paraquat. These data suggest a possible function for *PDX1.2* under stress conditions.

PDX1/PDX2 PLP synthase protein complex

A number of studies (including some described above) have characterized the interaction between PDX1 and PDX2 in what is known as the pyridoxal synthase complex (Fig. 4). As stated earlier, in this complex, PDX2 functions as a glutaminase, transferring nitrogen from glutamine to the pyridoxal molecule, and PDX1 functions as a ring closure protein. Twelve PDX1 and twelve PDX2 proteins form a large multimeric synthase complex (Strohmeier et al., 2006; Zein et al., 2006). In this complex, the twelve PDX1 monomers form a ring, and the PDX2 proteins bind to the PDX1 proteins to form an additional ring structure surrounding the PDX1 ring. The PDX2 proteins do not interact with each other (Strohmeier et al., 2006; Zein et al., 2006). It has been suggested that binding of PDX2 to PDX1 changes the

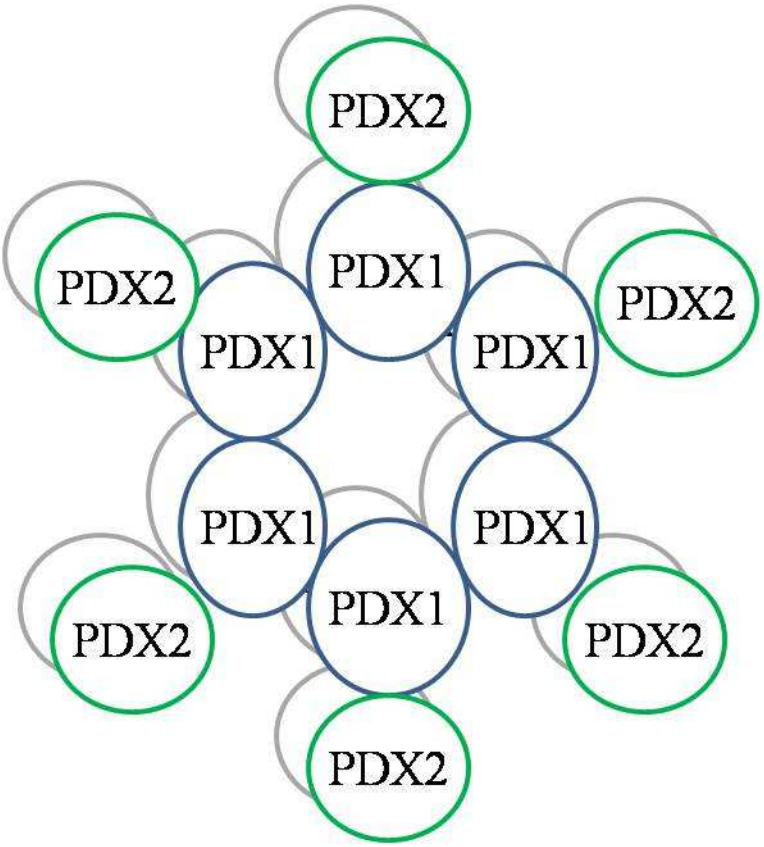

Figure 4. Schematic representation of PLP Synthase complex. The above figure represents the front half of the synthase complex, in which six PDX1 protein subunits interact to form an inner core, and a PDX2 protein subunit interacts with each PDX1 protein on the outside of the molecule. While PDX1 subunits have been shown to interact with each other and with PDX2 proteins, PDX2 proteins do not interact with other PDX2 proteins. The rear half of the PLP synthase complex (shaded gray) is identical and is located behind the front half, resulting in a complete synthase complex with a total of twelve PDX1 subunits and 12 PDX2 subunits

conformation of the PDX1 protein, enabling the PDX1 protein to catalyze synthesis of PLP (Raschle et al., 2009). The glutaminase activity is physically separated from the ring closure activity of the synthase portion of the complex and binding of

ribose5'-phosphate induces a conformational change in PDX1 subunits and increases cooperativity within the PDX1 ring structure. Additional binding of PDX2 and glutamine increases affinity of the synthase molecule for ribose 5'-phosphate. The PDX1 C-terminus may act as a lid, shielding or allowing access to the active site of an adjacent PDX1 monomer, providing a method of regulation of the synthase complex (Raschle et al., 2009). The location of the PDX1/PDX2 protein complex in cells is controversial. Analyses of protein sequences reveal no clear trafficking signals, and experimental evidence is inconsistent. Several different groups have attempted to experimentally localize this protein complex within the cell using GFP fusion proteins. Tambasco-Studart et al reported that all PDX1 proteins and PDX2 localize to the cytoplasm (Tambasco-Studart et al., 2005), however Chen and Xiong localized PDX1.3 to organelles and the membrane system (Chen and Xiong, 2005; Tambasco-Studart et al., 2005). In separate work, Denslow et al. also reported that the PDX2 protein canbe found at the plasma membrane and the nucleus, which supports the PDX1.3 localization reported by Chen and Xiong (Denslow et al., 2007). Information on subcellular localization compiled by the SUBA Arabidopsis Subcellular Localization Database (http://www.plantenergy.uwa.edu.au/webpages/research/databases.html) agrees with the GFP fusion studies in predicting PDX1 and PDX2 localization in both the cytosol and in organelles. Depending on the prediction program, PDX1.1, PDX1.3 and PDX2

are all predicted to localize in the cytosol, mitochondria and plastids; PDX1.2 is predicted to localize in the cytosol and mitochondria.

Salvage pathway of A. thaliana

As shown in Figure 1, vitamin B_6 is actually a collection of six different related molecules: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and the phosphorylated derivatives, PNP, PLP and PMP respectively. These vitamers are intercoverted between each other by a set of enzymes which comprise what is termed the "salvage pathway". While PLP is the dominant co-enzyme form, the other vitamers maintain a homeostasis within the cell through the salvage pathway. Phosphorylated vitamers are de-phosphorylated by a variety of phosphatases. The non-phosphorylated vitamers are phosphorylated by the PN/PL/PM kinase, and in the case of PL, also a specific PL kinase (PL). In addition to the kinases and the phosphatases, the salvage pathway also requires an oxidoreductase and a reductase. PDX3, pyridoxine (pyridoxamine) 5'-phosphate oxidase, catalyzes the formation of PLP from either PNP or PMP. Also, a pyridoxal reductase (PLR) that reduces PL to PN has also been described. Descriptions of the salvage enzymes are below.

Pyridoxal kinase

Pyridoxal kinase (EC 2.7.1.35) has been studied from a variety of organisms and tissues, including *Streptococcus faecalis, E. coli,* yeast, and brain and liver tissue (McCormick and Snell, 1959; Macaione et al., 1976; Spector and Shikuma, 1978; Yang et al., 1996; Yang et al., 1998; Shi and Zhu, 2002; Said et al., 2003; Di Salvo et al., 2004; Morita et al., 2004; Safo et al., 2004; Wrenger et al., 2005; Said et al., 2008). The enzyme is referred to by several other names, including pyridoxal 5'-phosphate-kinase, pyridoxal phosphokinase and pyridoxine kinase. Pyridoxal kinase activates the pyridoxine vitamers by phosphorylating them. It is a member of the phosphofructokinase enzyme family and as such, functions to transfer a phosphate group from ATP to the alcohol group of non-phosphorylated forms of vitamin B_6. Pyridoxal kinase can utilize several different cations, including magnesium (Mg^{2+}), cobalt (Co2+), manganese (Mn^{2+}) and zinc (Zn^{2+}) (McCormick et al., 1961).

The structure of pyridoxal kinase from *E. coli* has been elucidated. The protein has been shown to form a homodimer complex, with an active site on each subunit (Safo et al., 2004). The structure of pyridoxal kinase isolated from sheep brain is similar, and has also been shown to be composed of two pyridoxal kinase enzymes, which form a homodimer complex (Li et al., 2002). Although pyridoxal kinase is a member of the ribokinase superfamily, Li et al. have shown that this

enzyme lacks a lid-shaped structure common in other enzymes found in the ribokinase superfamily (Sigrell et al., 1998; Li et al., 2002).

PL (PN,PM) kinase

Several different pyridoxal kinase enzymes have been characterized in a variety of organisms. However, in *A. thaliana*, only one pyridoxal kinase has been characterized. PL(PN,PM) kinase (At5g37850) is a general pyridoxal kinase that phosphorylates all non-phosphorylated vitamers. It is homologous to the pdxK protein first characterized in *E. coli.* At5g37850 is also known as SOS4, and mutants of this protein were first characterized as a *salt overly sensitive* mutant (Shi and Zhu, 2002). Although this protein has not been experimentally localized in *A. thaliana*, it is predicted to be located in plastids and mitochondria. Macaione et al. showed pyridoxal kinase activity was present in mitochondrial fractions of ox retina (Macaione et al., 1976), and Huh et al., through the use of GFP fusion constructs, showed that in yeast, pyridoxal kinase is located in the cytoplasm and in the nucleus (Huh et al., 2003).

Several different papers have been published regarding SOS4 in *A. thaliana* (Shi and Zhu, 2002; Gonzalez et al., 2007). While searching for mutants hypersensitive to salt, Shi and Zhu identified *sos4*, and showed that root growth of these mutants was severely decreased in the presence of exogenous NaCl. In addition,

Shi and Zhu also reported that this gene is required for proper root hair growth in the maturation zone. GUS-promotor studies indicate that this gene is expressed in root tips.

Gonzalez et al. characterized the function of this gene in the vitamin B_6 salvage pathway (Gonzalez et al., 2007). Mutants of SOS4 are chlorotic and have a decrease in plant mass in comparison to WT plants, and show root sensitivity to exogenous sucrose in addition to the previously characterized salt sensitivity. Mutants of this enzyme accumulate higher levels of vitamin B_6 than their WT counterpart. The bulk of this extra accumulation can be attributed to higher levels of PLP, the active vitamer form, in addition to extra pyridoxine and pyridoxamine. The higher levels of PLP were unexpected, and may be due in part to upregulation of de novo pathway genes, as transcript accumulation of *PDX* genes was shown to be higher in *sos4* mutants than in WT plants. In addition to salt and sucrose sensitivity, Gonzalez et al. showed that mutants of SOS4 are more tolerant to drought and less tolerant to chilling than WT plants.

PL-specific kinase

In addition to PL(PN,PMP) kinase, a specific protein kinase has been characterized in *E. coli* and is referred to as PDXY. This specific kinase only phosphorylates PL and does not phosphorylate the other non-phosphorylated forms of

the vitamers. This protein was first characterized in *E. coli* by Yang, et al. in 1998 (Yang et al., 1998). The protein is specific in its substrate for PL, and its activity has been shown to be less than the PdxK protein (Yang et al., 1998; Di Salvo et al., 2004). In addition to identifying this kinase, Yang et al. also showed that in *E. coli*, the *PdxY* gene was co-regulated with TyrS, the gene for aminoacyl-tRNATyr synthetase (Yang et al., 1998). PLP has been shown to be a required coenzyme for a variety of aminoacyl synthetases. Of particular importance to the plant community is that of glutamyl-tRNA synthetase, which is not only required for protein synthesis, but is also required for synthesis of chlorophyll (Vavilin and Vermaas, 2002).

Pyridoxine (pyridoxamine) oxidase

Another important enzyme in the pyridoxal salvage pathway is the PNP (PMP) oxidase, which converts both PNP and PMP to PLP. This enzyme was identified in 1980 in *E. coli* by McCormick and Merrill. They reported that this protein is cytosolic, and requires both O_2 and flavin mononucleotide (FMN), which is also known as riboflavin 5'-phosphate (McCormick and Merrill, 1980). Kinetic properties of this enzyme in rabbit liver were characterized in 1983 by Choi et al. (Choi et al., 1983). In this study, PNP (PMP) oxidase was able to oxidize either the primary amine or the primary alcohol of the vitamer family to form the same PLP substrate. Choi et al. reported that oxidation of PNP occurs through a binary complex "ping

pong" mechanism, and that PMP oxidation is accomplished by a ternary sequential mechanism. In both of these mechanisms, oxygen functions as the electron acceptor (Choi et al., 1983). This protein was purified from *E. coli* in 1995 by Zhao and Winkler (Zhao and Winkler, 1995). They confirmed that this protein is a homodimer, but noted that, unlike the mammalian form, the favored substrate of the in vitro enzyme is PNP and not PMP. In 2000, Safo et al. determined the crystal structure of the *E. coli* oxidase (Safo et al., 2000). They also confirmed that the enzyme is a homodimer, with two identical subunits. The monomer is composed of an eight stranded beta sheet surrounded by 5 alpha helices. Two FMN binding clefts are formed at the interface of the two interacting monomers.

In 2007, the *A. thaliana* gene encoding PNP(PMP) oxidase (*PDX3,* At5g49970) was independently identified by Sang et al. and Gonzalez et al. (Gonzalez et al., 2007; Sang et al., 2007). Sang et al. expressed this protein in *E. coli* and confirmed function of the enzyme through assay of PNP(PMP) oxidase activity (Sang et al., 2007). In addition, they showed that *S. cerevisiae* deficient in PNP(PMP) activity were sensitive to oxidative stress. Gonzalez et al. confirmed the identity of the *A. thaliana* locus by complementation of *E. coli pdxH* mutants (Gonzalez et al., 2007). They then characterized insertion mutants of this gene in *A. thaliana* and confirmed a decrease in oxidase activity in two separate insertion mutant lines (Gonzalez et al., 2007). The *pdx3* mutants accumulated less total

vitamin B_6, although there was no significant difference in the levels of specific vitamers as compared to levels in WT. The *pdx3* mutants were smaller than WT plants grown under standard growth chamber conditions. In addition, *pdx3* mutants that were grown under high light conditions (1000 μmol s^{-1} m^{-2}) were unresponsive to increased light; while WT plant growth increased under high light conditions, the *pdx3* mutants showed no increase in plant mass. The *pdx3* mutants did not differ from WT in response to chilling or to osmotic stress caused by salt or sucrose. The *pdx3* mutants had increased expression of *SOS4* as well as the de novo pathway genes, particularly *PDX1.1*. Although de novo pathway gene expression increased, the *pdx3* mutants had lower PDX1 activity as compared to either WT or the *sos4* mutant.

Pyridoxal reductase

Pyridoxal reductase (1.1.1.65) is also known as pyridoxal dehydrogenase (Guirard and Snell, 1988). Guirard and Snell purified this enzyme from Baker's yeast and characterized its properties. They found that the enzyme forms pyridoxine by the reduction of pyridoxal. This reaction is most favorable at the pH of 6.0 – 7.0 and requires NADPH for activity. The reaction is reversible; pyridoxine is oxidized to pyridoxal, and this reaction is optimal at a pH of 8.6. Even though the enzyme is

bidirectional in vitro, the reaction favors the formation of pyridoxine (Guirard and Snell, 1988).

In 1999, Nakano et al. cloned, purified and characterized the catalytic activity of pyridoxal reductase from *Schizosaccharomyces pombe* (Nakano et al., 1999). They also found that the reaction was reversible, but showed that the reduction of pyridoxal was most favorable between pH 6.5 to 7.5, and that the oxidation reaction occurred most readily in the pH range 7.5 – 8.5. Therefore, even though the oxidation reaction occurs in vitro, it is unlikely to occur in vivo. In 2004, Morita et al. disrupted the *PLR* gene in *S. pombe* (Morita et al., 2004). The mutant yeast was able to grow in synthetic medium lacking vitamin B_6, indicating that this protein is not required for viability of the organism. This mutant accumulated lower levels of total vitamers, as well as lower amounts of PMP. In addition, their results suggested that the *PLR* gene is involved in efflux of PL and/or PN from the cell.

Pyridoxal phosphatases

Two different forms of pyridoxal phosphatases have been shown to exist. Alkaline phosphatases (EC 3.1.3.1) function at a higher pH than acid phosphatases (EC 3.1.3.2) which function optimally at acidic or neutral pH. Alkaline phosphatases have been implicated in the maintenance of extracellular concentrations of PLP, while

acid phosphatases have been implicated in maintenance of intracellular concentrations of the vitamin (Coburn and Whyte, 1988).

In 1992, Fonda reported the purification and characterization of soluble acid pyridoxal phosphatase from human erythrocytes (Fonda, 1992). This enzyme had an optimal pH activity of 6.0-6.5. The enzyme was shown to be a dimer with a molecular weight of approximately 64,000 kDa and required Mg^{2+} for activity. The enzyme catalyzed the removal of phosphate from PLP, PNP and PMP. Enzymatic activity was highest with PLP, followed by PNP and PMP. PL and inorganic phosphate inhibited the reaction.

Subsequent work by Fonda and Zhang in 1995 revealed that the soluble acid phosphatase from erythrocytes is not only activated by Mg^{2+}, but also by Co^{2+}, Ni^{2+} and Mn^{2+} (Fonda and Zhang, 1995). Mn^{2+} and Zn^{2+} were shown to activate the enzyme at low concentrations, but inhibited the enzyme at higher concentrations. Fonda and Zhang also showed that PLP and Mg^{2+} bind randomly to the enzyme to facilitate catalysis, but that both must bind before any product is released.

In 2003, Jang et al. cloned pyridoxal phosphatase from cDNA of human brain tissue (Jang et al., 2003). This protein was 296 amino acids in length and had a predicted molecular mass of 31,698 Daltons. They also cloned pyridoxal phosphatase from cDNA from mouse tissue. The two proteins were 93% identical. The mouse protein was 292 amino acids long and had a predicted molecular mass of 31,698

Daltons. Jang et al. expressed the human pyridoxal phosphatase in *E. coli*, and found the kinetic properties of this enzyme to be similar to that previously isolated from human erythrocytes. Transcript of the pyridoxal phosphatase was found in a wide variety of tissues, with a high abundance found in brain tissue.

Transport of vitamin B_6

Transport of B_6 vitamers across cellular membranes has been studied extensively. This vitamin is needed in almost all cellular compartments, the mitochondria, nuclei and plastids, for a variety of metabolic activities. As stated earlier, the location of enzymes for de novo synthesis within cells is not fully understood. Further, animals and other organisms that do not synthesize B_6 de novo must have a way to transport the vitamin. PLP, the active form of vitamin B_6, is a charged molecule which does not readily diffuse across cellular membranes, and it has been known for many years that only the non-phosphorylated vitamers move across cell membranes. Vitamin B_6 transport has been studied in several different organisms and tissues. Vitamin B_6 transport mechanisms have been identified in prokaryotes and lower eukaryotes such as *Salmonella typhimurium, S. cerevisiae*, *S. faecalis*, and *Saccharomyces carlsbergensis* (Mulligan and Snell, 1976; Shane and Snell, 1976; Mulligan and Snell, 1977; Stolz and Vielreicher, 2003). In addition, transport of vitamin B_6 has also been studied in tissues of higher eukaryotes,

including human intestinal epithelial tissues, mammalian colonocytes, renal cells of rats, and the brain of rabbits (Spector and Greenwald, 1978; Zhang and McCormick, 1991; Said et al., 2003; Said et al., 2008). From these studies, two different models of vitamin B_6 transport have been identified: facilitated diffusion and active transport.

Diffusion of the vitamin B_6 has been described in several organisms, including lactic acid bacteria (*S. faecilis*), *Trypanosoma brucei brucei,* and in brain and choroid tissues of rabbits (Mulligan and Snell, 1977; Spector and Greenwald, 1978; Spector and Shikuma, 1978; Gray, 1995). In this model of transport, non-phosphorylated vitamers diffuse across plasma membranes and are phosphorylated by pyridoxal kinase after diffusion. In this diffusion model of transport, pyridoxal kinase "traps" non-phosphorylated vitamers and converts them to their phosphorylated derivatives, effectively serving to create a "sink" for non-phosphorylated vitamers. This sink drives diffusion of the non-phosphorylated vitamins across the membrane, where they are subsequently phosphorylated. Several different bioinformatic prediction models predict that the pyridoxal kinase protein is located in organelles, such as mitochondria and plastids (http://www.plantenergy.uwa.edu.au/suba2/). Pyridoxal kinase activity has been shown to be present in mitochondrial fractions of ox retina (Macaione et al., 1976), and pyridoxal kinase has been located in the cytoplasm and the nucleus of yeast through the use of full length proteins conjugated with GFP (Huh et al., 2003).

These data are consistent with the hypothesis that indicates that pyridoxal kinase is involved in transport of vitamin B_6 into cellular compartments by molecular trapping.

Another model of vitamin B_6 transport which has been described is active transport. Recently, three different types of transport proteins have been identified in yeast and bacteria. In 2003, Tpn1p, a vitamin B_6 transporter was identified in *S. cerevisiae* (Stolz and Vielreicher, 2003). This protein primarily transports PN, but is also capable of transporting PM and PL. Tpn1p has been localized to the plasma membrane and transports non-phosphorylated vitamers through a proton symport mechanism (Stolz and Vielreicher, 2003). In 2005, Bsu1p was identified in *S. pombe* (Stolz et al., 2005). Like Tpn1p, this transport protein also is a proton symporter; however, this protein appears to be evolutionarily unrelated to the transporter in *S. cerevisiae*. In addition to these two proteins, another type of vitamin transporter has recently been identified in a variety of different prokaryotes, utilizing the SEED comparative genomics platform. These findings were confirmed in *Streptococcus* and *Lactobacillus* bacteria. This protein has a high affinity for pyridoxine, is a member of the energy-coupling factor (ECF) family of transporters, and is unrelated to both Tpn1p and Bsu1p (Rodionov et al., 2009). To date, no transport proteins have been identified in higher order eukaryotes, including plants.

Diseases associated with vitamin B_6 deficiency

A number of human diseases and conditions have been linked to a deficiency of vitamin B_6 including impaired immune functions, where vitamin B_6 levels are correlated with levels of both interleukin-2 and lymphocytes, important components of the immune system (Meydani et al., 1991) and to cognitive impairments, such as Alzheimer's disease. While vitamin B_6 supplementation did not ameliorate or prevent progression of Alzheimer's, vitamin B_6 deficiency was associated with white matter lesions (WMLs) in individuals afflicted with Alzheimer's disease (Mulder et al., 2007). Other neurologic abnormalities that have been associated with vitamin B_6 deficiency include chronic pain, depression, Parkinson's disease, and epilepsy (Bernstein, 1990).

Recent work has revealed that vitamin B_6 deficiency is common in the United States (Morris et al., 2008). Looking at specific demographic groups, this deficiency was highest in elderly individuals (men and women over the age of 65), African American men, men who smoke and women of reproductive age, especially those who use oral contraceptives. Vitamin B_6 deficiency is also common in alcoholics, where supplementation has been shown to relieve peripheral neuropathy (Bernstein, 1990).

A variety of circumstances can result in vitamin B_6 deficiency. The most obvious of these circumstances is poor nutrition. Since humans and other animals

cannot synthesize vitamin B_6, they must obtain it from their diet. Vitamin B_6 can be obtained from nutrient rich foods, such as meats (pork or chicken), fish, bananas, legumes, green leafy vegetables and fortified foods such as breakfast cereals. Other causes of deficiency include interactions with medications and other substances ingested by the body. Vitamin B_6 binds to several different types of medication, including isoniazid and cycloserine, which is used to combat tuberculosis (Biehl and Vilter, 1954; Devadatta et al., 1960) and penicillamine, which is a chelating agent used to remove toxic molecules from the body (Rothschild, 1982).

While many different aspects of vitamin B_6 metabolism have been characterized, there is still much to learn about vitamin B_6 metabolism. New functions of vitamin B_6 are continuing to be discovered, such as its ability to regulate gene function by binding to transcriptional regulators (Cavailles et al., 1995; Huq et al., 2007). More research is needed to completely understand the function of vitamin B_6 and its role in cellular metabolism. Most of the characterization of function of this metabolite has been done in animals and bacteria. Recently, progress has been made in the characterization of vitamin B_6 metabolism in plants, particularly in *A. thaliana.* Several phenotypes in pathway mutants have been described, but are not explained by known roles of vitamin B_6 metabolism. A great deal remains to be discovered about vitamin B_6 in plants, particularly in characterization of unexplained phenotypes

produced by changes in levels of vitamin B_6, characterization of the salvage pathway enzymes and in transport of vitamin B_6 across membranes and tissues.

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Chapter 2: Regulation of the *Arabidopsis thaliana* vitamin B_6 biosynthesis genes by abiotic stress

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Research article

Regulation of the *Arabidopsis thaliana* vitamin B_6 biosynthesis genes by abiotic stress

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Abstract

Vitamin B_6 (pyridoxine and its vitamers) plays an essential role as a co-factor for enzymatic reactions and has also recently been implicated in defense against cellular oxidative stress. The biosynthetic pathway was thoroughly characterized in *Escherichia coli*, however most organisms, including plants, utilize an alternate pathway involving two genes, *PDX1* and *PDX2*. *Arabidopsis thaliana* contains one copy of *PDX2*, but three full-length copies of *PDX1*, one each on chromosomes 2, 3, and 5 (referred to as *PDX1.1*, *PDX1.2*, and *PDX1.3*, respectively). Phylogenetic analysis of the PDX1 homologues in *A. thaliana* showed that PDX1.1 and PDX1.3 clustered with the homologues from the other dicots, whereas PDX1.2 was more divergent, and did not cluster with either the dicots or monocots. Expression analysis using quantitative PCR showed that *PDX1.1* and *PDX1.3* were highly expressed in *A. thaliana* rosettes, while *PDX1.2* showed only low level expression. All three *PDX1* genes and *PDX2* were responsive to abiotic stressors including high light, chilling, drought, and ozone, however, the response of *PDX1.2* was disparate from that of the other *PDX* genes, showing a lessened response to high light, chilling, and drought, but an increased response to ozone. Green fluorescent protein fusion studies demonstrated that PDX2 localizes in the nucleus and membranes of cells, consistent with recent published data for PDX1. Insight into regulation of the biosynthetic genes during abiotic stress could have important applications in the development of stress-tolerant crops.

Keywords: Antioxidant; Plant abiotic stress; *SNZ1*; *SNO*; *YaaD*; *YaaE*

1. Introduction

Vitamin B_6, in its active form pyridoxal 5′-phosphate, is an essential co-factor most notable for its involvement in amino acid biosynthesis. The pathway for synthesis of the B_6 vitamers (pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives) has been thoroughly characterized in *Escherichia coli* [16,22]. However, our laboratory and others recently documented that the majority of B_6-producing organisms, including plants, fungi, archaebacteria, and many bacteria, use a distinct pathway for *de novo* production involving two genes, *PDX1* and *PDX2* [17,18,26]. Recent work, primarily with *PDX1* and *PDX2* homologues from *Bacillus subtilis* (*YaaD* and *YaaE*) and *Saccharomyces cerevisiae* (*SNZ and SNO*), has led to the characterization of the pathway enzymes and intermediates [3,7,15,29,39]. These studies showed that the PDX1 and PDX2 proteins form a complex having glutamine amidotransferase activity, with the PDX2 protein serving as a glutaminase. The reaction utilizes glutamine plus either ribose 5-phosphate or ribulose 5-phosphate and either dihydroxyacetone phosphate or glyceraldehyde 3-phosphate and produces pyridoxal 5′-phosphate. This reaction is distinct from that in *E. coli* and other proteobacteria which utilize glutamate, 1-deoxy-D-xylulose 5-phosphate

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and 4-phosphohydroxy-L-threonine and produce pyridoxine 5′-phosphate [22].

Very recently, *PDX1* and *PDX2* homologues from plants have been isolated and characterized [9,14,36,37]. *Nicotiana tabacum* contains a single copy of *PDX2*, but two copies of *PDX1*, which have identical predicted amino acid sequences but differ by 15 nucleotides [14]. In *Arabidopsis thaliana*, there is also a single copy of *PDX2*, but three full-length copies of *PDX1* on chromosomes 2, 3, and 5 [36]. Reconstitution experiments with recombinant proteins confirmed the substrates and product were identical to those reported for *B. subtilis*, and mutant analysis documented that *PDX2* is essential for viability [36]. Analysis of expression of the *PDX* genes in *A. thaliana* showed that *PDX2* was expressed equally well in all tissues, and that the *PDX1* homologue on chromosome 2 was expressed primarily in leaves whereas the chromosome 3 *PDX1* homologue was expressed primarily in roots [37]. Studies on the expression of the chromosome 5 *PDX1* homologue by two independent groups showed expression in most tissues; however, the studies differed on expression of this homologue in roots [9, 37]. Two reports characterizing the cellular localization of the *A. thaliana* proteins differed in their findings, with one study showing that all proteins were cytoplasmic [36], and the other demonstrating membrane localization of the protein encoded by the chromosome 5 homologue [9].

In addition to the identification of the novel pathway for B_6 biosynthesis, recent information has also uncovered that vitamin B_6 vitamers, similar to vitamins C and E, show antioxidant activity and may be an important component of cellular antioxidant defenses. We have shown that B_6 vitamers are efficient quenchers of both singlet oxygen and superoxide and have antioxidant activity [4,14]. B_6 vitamers have also been shown to quench superoxide and prevent lipid peroxidation and protein oxidation in blood due to high glucose and H_2O_2 [23,24,33]. Pyridoxine delayed and attenuated active oxygen-dependent plant defense responses to a bacterial pathogen [14] and reduced singlet oxygen-induced death in *A. thaliana flu* mutant protoplasts [12]. *A. thaliana* mutants deficient in the *PDX1* homologue on chromosome 5 were shown to be highly sensitive to osmotic and oxidative stresses and had increased lipid peroxidation in response to UV treatment [9]. In clinical trials, vitamin B_6 supplements prevented or delayed eye and nerve damage associated with diabetes and attributed to superoxide production [23].

Vitamin B_6 has also been linked to oxidative stress in diverse organisms through gene regulation studies of both the *de novo* biosynthetic genes (*PDX1* and *PDX2*) as well as the genes encoding enzymes in the salvage pathway (pyridoxal kinase, oxidase, and reductase), which interconvert between the different vitamers. For example, in *B. subtilis*, the *PDX1* homologue shows increased expression following treatment with the superoxide generator paraquat [2]. Hydrogen peroxide treatment of *Schizosaccharomyces pombe* led to increased transcript abundance of both *PDX2* and the gene encoding pyridoxal reductase [8]. *S. cerevisiae* showed 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 [5]. In *A. thaliana*, UV-B radiation led to increased transcript of the *PDX1* homologue on chromosome 5 [6], and *PdxK* (encoding pyridoxal kinase) showed increased transcript during cold stress [31]. Finally, our laboratory and others showed that salicylic acid, methyl jasmonate, and ethylene, chemical inducers of oxidative plant defense responses, increase *PDX* transcript in *N. tabacum* and *Hevea brasiliensis* (rubber tree) [14,32].

While plants produce active oxygen species (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 affects 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, α-tocopherol, flavonoid pigments, and carotenoids, and proteins with repair functions (heat shock proteins) [13,21,27,38]. The goal of this work was to determine if vitamin B_6 may also serve as a cellular defense against abiotic stress-induced AOS by determining if the *de novo* vitamin B_6 pathway genes in *A. thaliana* are regulated in response to abiotic stress. Here we show via quantitative reverse transcription PCR (qPCR) that the *A. thaliana PDX1* and *PDX2* genes are up-regulated by abiotic stressors including high light, chilling, drought, and ozone, suggesting that vitamin B_6 plays an important role in oxidative stress responses in plants. Further, in agreement with Chen and Xiong [9] and in contrast to Tambasco-Studart et al. [36], our work supports the localization of the PDX enzyme complex in the plasma membrane of cells, as well as in the nucleus.

2. Results

2.1. Identification and comparison of B_6 biosynthetic genes in A. thaliana Columbia-O ecotype

Homologues to vitamin B_6 biosynthetic genes, *PDX1* and *PDX2*, were identified in *A. thaliana* Colombia-O ecotype by sequence homology to known *PDX1* and *PDX2* sequences from the fungi *Cercospora nicotianae* and *S. cerevisiae* using GenBank and TAIR databases. In agreement with the results of Tambasco-Studart et al. [36] we identified three full-length copies of *PDX1*, one each on chromosomes 2, 3, and 5 [referred to as *PDX1.1* (At2g38230), *PDX1.2* (At3g16050), and *PDX1.3* (At5g01410), respectively]. *PDX1.1* and *PDX1.3*, the more similar of the three homologues, each encode proteins of 309 amino acids in length that are 89% identical. *PDX1.2*, encodes a slightly longer protein of 314 amino acids, with an extended amino terminus as compared to PDX1.1 and PDX1.3. PDX1.2 is only 60% identical to PDX1.1 and PDX1.3. All three *PDX1* genes have open reading frames composed of one continuous exon, as is found for all *PDX1* homologues identified to date [5,14,19,28]. A partial copy of *PDX1* of 240 nucleotides was also identified on chromosome

2; it is 100% identical to the first third of *PDX1.1* and is located 4 kb away. We identified one copy of *PDX2* on chromosome 5 (At5g60540). *PDX2* contains 6 exons and codes for a protein of 255 amino acids.

We conducted parsimony analysis of the three full-length copies of PDX1 (Fig. 1). While PDX1.1 and PDX1.3 group with PDX1 sequences from other dicots, PDX1.2 is clearly divergent. Although the PDX1.2 sequence does group with the plant sequences, and is separate from the fungal sequences, it does not group with either dicots or monocots. This divergence is supported by boot strap analysis.

Quantitative PCR was used to compare relative transcript abundance of the three *PDX1* genes and *PDX2* in *A. thaliana* rosettes. While *PDX1.1* and *PDX1.3* transcripts were present in high amounts, *PDX1.2* transcript showed only low level abundance, at less than 0.5% of *PDX1.1* or *PDX1.3* (Fig. 2A). *PDX2* transcript also showed low level abundance, but is still more prevalent than *PDX1.2*. Data obtained from the Genevestigator website [41] confirms our qPCR data (Fig. 2B). Further, Genevestigator data show that array expression levels for *PDX1.1* and *PDX1.3* are high compared to the average expression of all genes (data not shown).

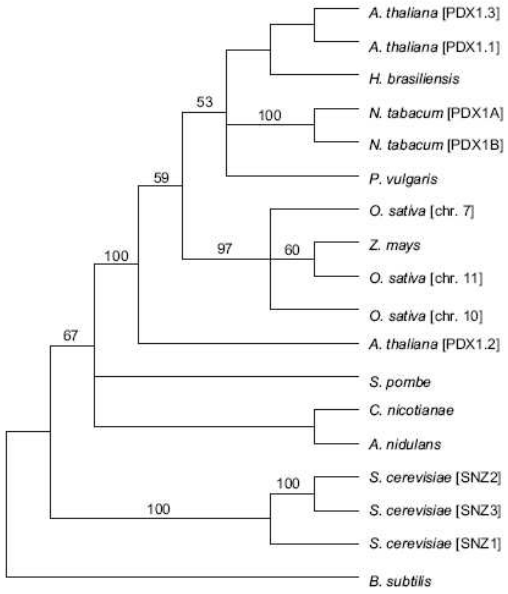

Fig. 1. Parsimony analysis of PDX1 proteins. Diagram shows the strict consensus tree of plant and fungal PDX1 sequences using *Bacillus subtilis* to root the tree. Bootstrap values (>50%) are indicated above branches. Species and GenBank accession numbers: *A. thaliana*, O80448; *A. thaliana*, Q9ZNR6; *Hevea brasiliensis*, Q39963; *Nicotiana tabacum*, AY532656.1; *N. tabacum*, AY532657.1; *Phaseolus vulgaris*, Q9FT25; *Oryza sativa*, AP008213.1; *Zea mays*, AY106100 (modified); *O. sativa*, AP008217.1; *O. sativa*, AC108883.1; *A. thaliana*, Q9ZNR6; *Schizosaccharomyces pombe*, NM_001020413.1; *Cercospora nicotianae*, O59905; *Aspergillus nidulans*, XM_675902.1; *Saccharomyces cerevisiae*, P53824; *S. cerevisiae*, P43545; *S. cerevisiae*, Q03148; *Bacillus subtilis*, P37527.

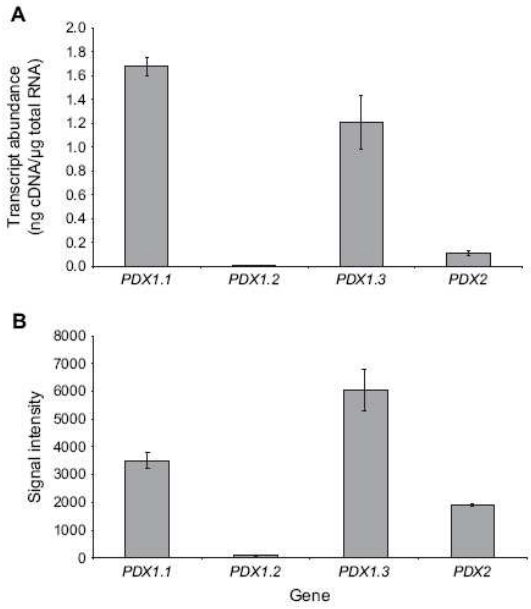

Fig. 2. Comparison of transcript abundance of vitamin B_6 biosynthetic genes. (A) Quantitative PCR showing transcript abundance of B_6 biosynthetic genes in *A. thaliana* rosettes under normal growth conditions. Transcript abundance shown as ng cDNA per μg total RNA. All samples were normalized to 18S rRNA. Error bars = standard error. (B) Transcript abundance of B_6 biosynthetic genes from *A. thaliana* obtained from published array data using Genevestigator. Transcript abundance shown as array signal intensity. Error bars = standard error. Our data agree closely with those from Genevestigator showing that *PDX1.1* and *PDX1.3* are the most highly expressed, with *PDX1.2* showing very low expression. Expression of *PDX2* was intermediate.

2.2. B_6 biosynthetic gene regulation by abiotic stress

The effect of various environmental and oxidative stresses on transcript abundance of the *PDX1* genes and *PDX2* was tested using qPCR. For high light stress, plants grown in a growth chamber under 200 μmol s^{-1} m^{-2} light were transferred to 1000 μmol s^{-1} m^{-2} light and transcript accumulation in rosettes was measured after 1, 4, and 7 days of stress treatment. Results are shown in Fig. 3, where transcript abundance is shown as fold change of high light-treated plants over control plants that were maintained at 200 μmol s^{-1} m^{-2} light. *PDX1.1*, *PDX1.2*, *PDX1.3*, and *PDX2* all showed a similar pattern in transcript abundance reaching the highest fold change over controls early in the stress treatment (day 1), followed by a return to control levels or below on day 4, and then another, smaller increase above control levels on day 7 of treatment (Fig. 3A). Although all four genes showed the same pattern of response, the response on day 1 for *PDX1.2* 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 (Fig. 3B).

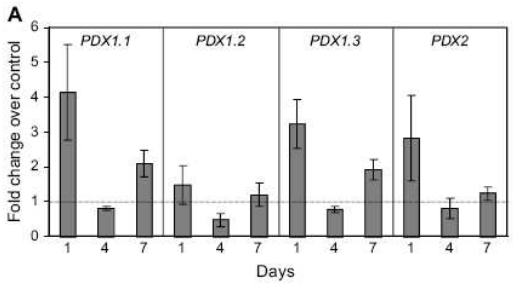

Fig. 3. Transcript abundance of B_6 biosynthetic genes after high light stress. (A) Quantitative PCR showing transcript abundance of B_6 biosynthetic genes in *A. thaliana* rosettes after increasing light strength from 200 to 1000 μmol s^{-1} m^{-2} for 1, 4, and 7 days. Transcript abundance shown as the average fold change in light-stressed plants (1000 μmol s^{-1} m^{-2} light) over control treated plants (200 μmol s^{-1} 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 were normalized to 18S rRNA. Error bars = standard error. (B) *A. thaliana* after 7 days of control treatment (200 μmol s^{-1} m^{-2}) (left) or high light treatment (1000 μmol s^{-1} m^{-2}) (right). Purple color is due to increased production of anthocyanins.

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 qPCR 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 Fig. 4. 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. *PDX1.1* showed the greatest increase in transcript abundance compared to control levels with an average 8-fold change. *PDX1.3* and *PDX2* also showed large fold changes after one day of cold stress. Again, similar to what was seen with light stress, *PDX1.2* showed the least amount of change compared to control treatments, reaching only an average 1.8-fold increase on day 1 of treatment. Chilling stress visibly affected the plants causing reduced growth and increased anthocyanin production (not shown).

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. After 7 days of drought stress, all four genes showed no change or a slight increase in transcript abundance compared to control plants (Fig. 5). By 14 days, *PDX1.1*, *PDX1.3*, and *PDX2*, but not *PDX1.2*, showed a slight, but significant increase in transcript abundance compared to controls of about 1.8-fold. Transcript abundance for *PDX1.2* was unaffected at day 14, remaining near control levels.

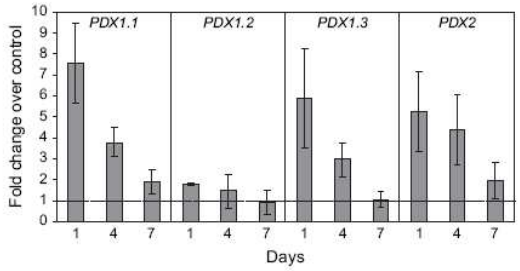

Fig. 4. Transcript abundance of B_6 biosynthetic genes after chilling stress. Quantitative PCR showing transcript abundance of B_6 biosynthetic genes in *A. thaliana* rosettes after decreasing temperature from 20 °C to 5 °C for 1, 4, and 7 days. Transcript abundance shown as the average fold change in 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 were normalized to 18S rRNA. Error bars = standard error.

The direct effect of reactive oxygen species was tested by treating *A. thaliana* with ozone and hydrogen peroxide. For ozone treatment, plants were exposed to 8 h per day of 125 ppb ozone or air control for 1 and 2 days. qPCR showed three distinct responses (Fig. 6). For both time points, *PDX1.1* transcript abundance decreased, with transcript levels dipping to half that found in control plants after one day of treatment. By contrast, *PDX1.2* showed a consistent increase in transcript abundance compared to controls, reaching a 3-fold increase on day 1 of ozone treatment. *PDX1.3* 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.

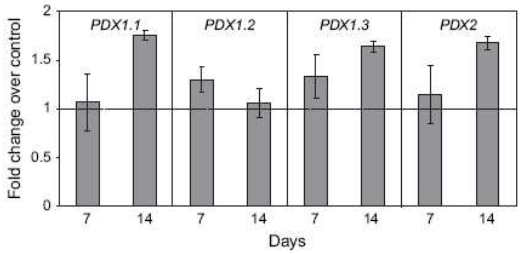

Fig. 5. Transcript abundance of B_6 biosynthetic genes after drought stress. Quantitative PCR showing transcript abundance of B_6 biosynthetic genes in *A. thaliana* rosettes after withholding water for 7 and 14 days. Transcript abundance shown as the average fold change in drought stressed plants (no 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 were normalized to 18S rRNA. Error bars = standard error.

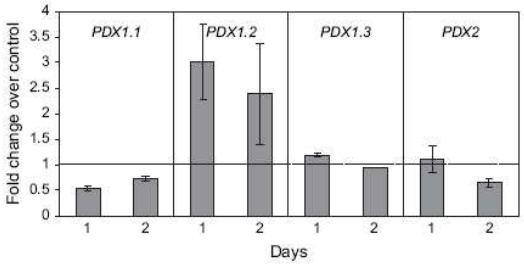

Fig. 6. Transcript abundance of B_6 biosynthetic genes after ozone stress. Quantitative PCR showing transcript abundance of B_6 biosynthetic genes in *A. thaliana* rosettes after 1 and 2 days of ozone treatment (125 ppb). Transcript abundance shown as the average fold change in ozone-stressed plants over control plants from three experiments. Transcript abundance in control treated plants is represented by the dotted line at a fold change of one. All samples were normalized to 18S rRNA. Error bars = standard error.

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 h after treatment, but all four showed a slight increase 3 h after treatment (data not shown). *PDX1.3* showed the most change in transcript abundance from control with an average 2.2-fold change 3 h after treatment, followed by *PDX1.1* with an average 1.6-fold change in transcript abundance over control 3 h after treatment. *PDX1.2* and *PDX2* showed the least change, both with an average 1.3-fold change in transcript abundance over control at 3 h after treatment.

Publicly available microarray data confirm that the three copies of *PDX1* and *PDX2* are differentially regulated by abiotic stressors. Genevestigator shows that after paraquat and UV-B stress, expression levels of *PDX1.1*, *PDX1.3* and *PDX2* remain unchanged from control levels (Fig. 7). *PDX1.2* expression, however, changed in response to both showing a three to fivefold increase at 6, 12 and 24 h after 10 μM paraquat treatment and more than a tenfold change 6 h after a 15 min UV-B treatment (Fig. 7; [41]).

2.3. Localization of enzyme complex in cells

Abiotic stresses such as chilling and high light lead to membrane damage. Thus the ability of vitamin B_6 to protect against abiotic stress may be impacted by the location of vitamin production in cells. Previous work by Chen and Xiong [9] and Tambasco-Studart et al. [36] came to differing conclusions on the localization of the PDX1 proteins. As PDX1 must form a complex with PDX2 for vitamin B_6 biosynthetic activity [7,29], we used green fluorescent protein fused to PDX2 as a marker for where the enzyme complex localizes in *A. thaliana*.

A. thaliana plants were transformed to express PDX2 fused to GFP or GFP alone. Expression of GFP and the GFP–PDX2 protein fusion was verified by western analysis, which demonstrated that the GFP–PDX2 fusion protein was intact in the leaves of transformed plants (Fig. 8). Fluorescence was

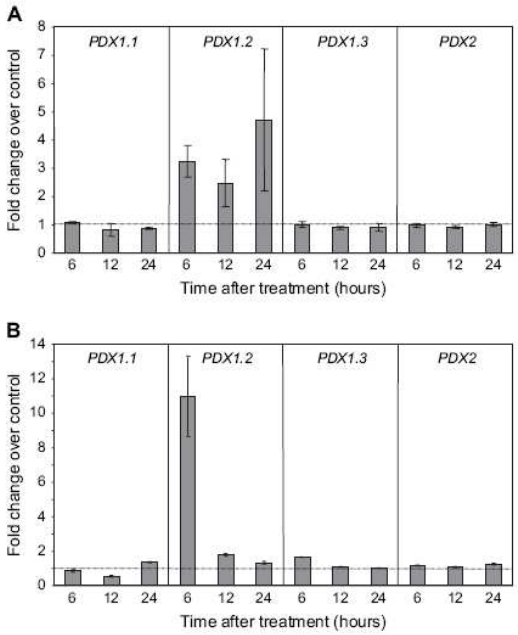

Fig. 7. Genevestigator data showing transcript abundance of B_6 biosynthetic genes after paraquat or UV-B stress. Transcript abundance of B_6 biosynthetic genes after 6, 12, and 24 h of treatment with 10 μM paraquat (A) or after 6, 12, and 24 h of recovery following a 15 min exposure to UV-B light (1.18 W m^{-2} Phillips TL40W2) (B). Transcript abundance shown as average fold change of experimental plants over control plants from two replications. Transcript abundance in control plants is represented by the dotted line at a fold change of one. Error bars = standard error. Data extracted from Genevestigator.

imaged in leaf epidermal peels. Compared to the generalized and diffuse fluorescence of GFP alone (Fig. 9F–H), fluorescence of the GFP–PDX2 fusion was observed at the periphery of epidermal cells (not shown) and guard cells as well as in the nuclei of guard cells (Fig. 9A–C). Plasmolysis in mannitol confirmed that the peripheral fluorescence was associated with the plasma membrane rather than the wall and also showed fluorescence associated with the cytoplasm (Fig. 9D,E). Our results with PDX2 agree closely with those of Chen and Xiong [9] for PDX1.3. Interestingly, PSORT analysis (http://psort.ims.u-tokyo.ac.jp/) of the PDX2 protein predicts localization in the membrane or the lumen of the endoplasmic reticulum and also in the peroxisomes.

3. 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* [7,17,29]. Only very recently has information been available on the pathway in plants [9,14,36,37]. We used homology to conserved sequences from

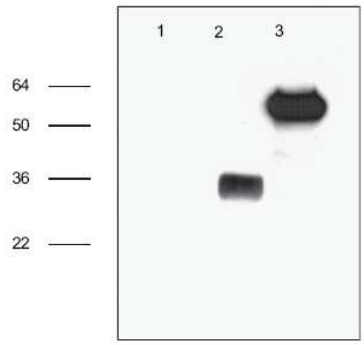

Fig. 8. Western analysis verifying expression of green fluorescent protein or green fluorescent protein fused to PDX2 in transformed *A. thaliana*. Lane 1: untransformed, wild type *A. thaliana*; lane 2: *A. thaliana* expressing GFP alone; lane 3: *A. thaliana* expressing GFP–PDX2 fusion protein. No GFP is detected in the untransformed *A. thaliana*. Bands of approximately 29 kDa and 53 kDa are observed in the GFP alone and GFP–PDX2 fusion lines, respectively.

fungal homologues to identify the three *PDX1* and one *PDX2* homologues in *A. thaliana* Columbia-O ecotype. Interestingly all three *PDX1* genes were composed of one uninterrupted exon, a characteristic that is true for all *PDX1* homologues identified to date from any organism. Lack of introns is a characteristic of genes for stress-related proteins, as splicing is affected by stress [40]. This result is consistent with our hypothesis of an important role for this pathway in stress responses. Although all three *PDX1* homologues were strongly conserved (as are all *PDX1* homologues thus far identified), *PDX1.2*, is more distinct from the others, showing less sequence identity and an elongated amino terminus. Phylogenetic analysis of PDX1 homologues showed that PDX1.1 and PDX1.3 clustered with the homologues from the other dicots, whereas PDX1.2 was more divergent, and did not cluster with either the dicots or monocots.

A difference between *PDX1.2* and the other two copies was also reflected in relative transcript expression levels of the three copies. Our qPCR studies demonstrated that *PDX1.1* and *PDX1.3* were highly expressed in *A. thaliana* rosettes, while *PDX1.2* showed only low level expression at less than 0.5% of *PDX1.1* or *PDX1.3*. Our studies also demonstrate that *PDX2* is not as highly expressed as *PDX1.1* or *PDX1.3*, although it is more highly expressed than *PDX1.2*. Our results are supported by data from Genevestigator but conflict with those of Tambasco-Studart et al. [36] whose qPCR studies showed *PDX2* having the highest level of expression, followed by *PDX1.3*, *PDX1.1*, and *PDX1.2*. Interestingly, Tambasco-Studart et al. [36] suggested that PDX1.2 was non-functional in pyridoxal phosphate biosynthesis due to its inability to complement a yeast *SNZ1* mutant and lack of activity in *in vitro* assays using recombinant proteins.

Vitamin B_6 has been shown to quench AOS and protect molecules from oxidative damage [4,9,12,14,23,24,33]. Chen and Xiong [9] showed that a mutant deficient in PDX1.3 was sensitive to salt, UV-B, and singlet oxygen stress. We have shown in *N. tabacum* that the B_6 biosynthetic genes are regulated in a manner consistent with a role of this vitamin as an antioxidant [14]. During a bacterially-induced hypersensitive defense reaction (which is dependent on AOS), the *N. tabacum PDX1* and *PDX2* genes were strongly downregulated, consistent with a need to reduce the amount of antioxidant compounds in the cells. Consistent with this result, we also showed that elevated pyridoxine delayed and attenuated the hypersensitive defense response and also increased symptom expression following inoculation with a virulent pathogen. Although vitamin B_6 is detrimental to plant defense responses against biotic pathogens, we hypothesized that this vitamin may protect against abiotic stress responses. Abiotic stresses, such as high light, low temperature, drought, salt, nutrient deprivation, ozone and UV irradiation all lead to an increased cellular oxidative stress [20,21,25,34].

We found that transcript abundance of all four biosynthetic genes was increased by different stress conditions; however, the pattern of accumulation differed for the stressors we tested. In general, *PDX1.2* diverged the most in its response pattern from the other *PDX1* genes and *PDX2*. While all four genes showed increased transcript abundance during high light and chilling stress, the increase for *PDX1.2* was much lower. Drought stress led to little if any change in *PDX1.2* transcript abundance, while showing an increase for *PDX1.1*, *PDX1.3*, and *PDX2*. And, while ozone stress did not increase transcript abundance for *PDX1.1*, *PDX1.3*, or *PDX2* (actually leading to a decrease in *PDX1.1* and *PDX2* transcript abundance), it led to a 3-fold increase in *PDX1.2* transcript. This differential response of *PDX1.2* to specific stresses is supported by data from Genevestigator, which showed *PDX1.2* alone being responsive to paraquat and UV-B. Taken together, our data suggest that PDX1.2 may have unique functions in the cell in response to specific stresses.

Interestingly, Chen and Xiong [9], while concluding that vitamin B_6 is critical for stress tolerance in plants, cited microarray data that the genes were not regulated by stress conditions. Our qPCR data conflict with their results and show that the B_6 biosynthetic genes are in fact regulated by diverse abiotic stressors. Our regulation data are consistent with their findings that *A. thaliana PDX1.3* mutants are sensitive to salt, UV, and generators of singlet oxygen, and together argue for an important role of this vitamin in stress responses in plants. Our data are supported by the array analysis results of Brosche et al. [6] who showed that UV-B radiation led to increased transcript of *PDX1.3*.

Two recent papers on the B_6 biosynthetic genes in *A. thaliana* came to conflicting information on the localization of the enzymes in cells. Both used green fluorescent protein fusions for this work. Tambasco-Studart et al. [36] used transient expression studies in onion epidermal cells and *A. thaliana* protoplasts, and argued that all three PDX1 proteins and PDX2 were cytoplasmic. By contrast, Chen and Xiong [9] transformed *A. thaliana* to express a PDX1.3-GFP fusion and showed that the protein was localized in the plasma membrane and other membranes of

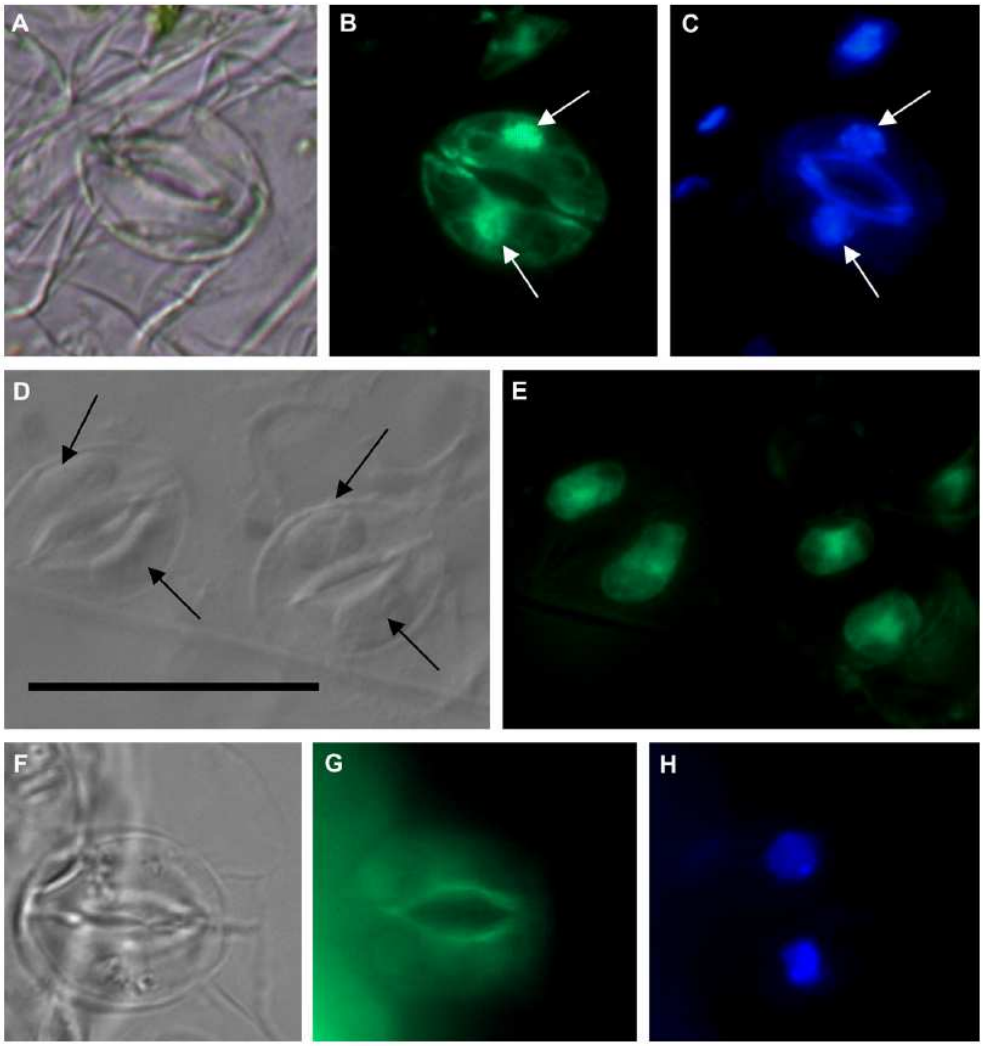

Fig. 9. Fluorescence microscopy of epidermal peels from leaves of *A. thaliana* transformed with green fluorescent protein alone or PDX2 fused with green fluorescent protein. (A, B, C) Phase, GFP fluorescence, and DAPI staining, respectively, of a guard cell from a fixed leaf epidermal peel expressing GFP–PDX2. Fluorescence is visible at the guard cell periphery and in the nuclei (arrows). (D, E) Plasmolyzed guard cells (black arrows, panel D) following treatment of an unfixed leaf peel in 100 mM mannitol. Fluorescence at the periphery of the cells is associated with the plasma membrane and not with the guard cell wall, and is also evident in the cytoplasm. (F, G, H) Phase, GFP fluorescence, and DAPI staining, respectively, of a guard cell from a fixed leaf epidermal peel expressing GFP alone. Bar = 50 μm.

the cell, including the nuclear envelope and outer chloroplast membranes, with a small amount associated with the cytoplasm. As PDX1 proteins must form a complex with PDX2 to be functional in vitamin B_6 biosynthesis, we used PDX2–GFP fusions to localize the enzyme complex in cells. Our data are most consistent with the results of Chen and Xiong [9]. We showed that PDX2 localizes in the plasma membrane of cells and also in the nucleus, with lesser amounts in the cytoplasm evident when the cells were plasmolyzed. Chen and Xiong [9] also identified PDX1.3 as localizing in membranes with lesser amounts visible in the cytoplasm when cells were plasmolyzed. As many of the stresses, including chilling and high light, damage membranes in cells, localization of the enzyme machinery in membranes would increase the efficacy of this vitamin as serving to protect against these stresses.

In summary, we have shown that the *de novo* vitamin B_6 biosynthetic genes, *PDX1* and *PDX2*, in *A. thaliana* are up-regulated, though differentially, by diverse abiotic stressors including high light, chilling, drought, ozone, and hydrogen peroxide treatments. These results support previous work by us and others that this vitamin serves as a cellular antioxidant and plays an important role in oxidative stress responses in plants and other organisms. Further, our work confirms the data of Chen and Xiong [9] and conflicts with that of Tambasco-Studart et al. [36] that the enzyme complex is localized within membranes in plant cells, a site that would enhance the ability of this vitamin to serve as a protector against cellular oxidative stress. We conclude that vitamin B_6 is important as an antioxidant during stress *in planta*.

4. Materials and methods

4.1. Sequence identification and analysis

Homologues to *PDX1* and *PDX2* were identified in *A. thaliana* ecotype Columbia-O by sequence homology to known *PDX1* and *PDX2* sequences from *C. nicotianae* and *S. cerevisiae* using blast searches through NCBI and TAIR databases [1,30]. Sequences were analyzed using Vector NTI. *A. thaliana* protein sequence alignment was performed using clustalW [10]. For phylogenetic analysis, protein sequences (identified in Fig. 1) were first aligned using clustalX. The sequence for *Zea mays* was altered such that the guanine nucleotide at position 486 in the mRNA sequence was deleted; the deletion allowed for greater homology between the predicted amino acid sequence and other PDX1 sequences. Phylogenetic relationships were identified through parsimony analysis by a full heuristic search with 100 replicates and rooting to *B. subtilis* (PAUP 4.0b10) [35]. To evaluate clade support, 1000 replicates of bootstrap analysis were performed using full heuristic search.

4.2. Plant growth and stress treatment

A. thaliana ecotype Columbia-O (Lehle Seed, Round Rock, TX) was grown on *Arabidopsis* growth medium (Lehle seed) at 20 °C, under an 8 h photoperiod (08:00–16:00 h) at 80 μmol s^{-1} m^{-2} light in a Percival growth chamber. Plants were watered 2–3 times per week, fertilizing every 2 weeks. Plants were stressed at 5–6 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 h. 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 h photoperiod [08:00–16:00 h], at 200 μmol s^{-1} m^{-2} light) 2 weeks prior to the experiment. High-light experiments were conducted at 1000 μmol s^{-1} m^{-2} light, and chilling experiments were conducted at 5 °C. For ozone stress, plants were exposed to 125 ppb ozone or air control. For all treatments, plants were transferred to treatment chambers (if applicable) at 16:00 h on day 0 and samples were taken at the appropriate day at 15:30 h. Controls were collected at the same time points as stress-treated plants. For each treatment and time point, rosettes from three plants were collected, bulked and immediately frozen in liquid nitrogen. Tissue was stored at −80 °C until used for RNA extractions.

4.3. RNA isolation and reverse transcription

Total RNA was extracted from leaf tissue using Tri-Reagent (Sigma, St. Louis, MO) 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 h. RNA was reverse transcribed in a ratio of 20 ng per 1 μl 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.

4.4. Quantitative PCR

PCR was performed using 5 μl cDNA mix (corresponding to 100 ng starting total RNA) in 25 μl total volume of reaction mix containing SYBR Green Mastermix (Applied Biosystems) and gene specific primers (0.8 pmol μl^{-1} final concentration). Primers (Integrated DNA Technologies, Coralville, IA) are listed in Table 1. 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). Standard curves were generated using known

Table 1
Primers for *de novo* vitamin B_6 biosynthetic genes in *A. thaliana* ecotype Columbia used for qPCR and GFP vector construction

Gene	Forward primer	Reverse primer
PDX1.1 qPCR	5′-TCTCCCTTCTCCGTGAAAGTTG-3′	5′-GCGTTGACGACATCCATGATT-3′
PDX1.2 qPCR	5′-AGGTCGGATTAGCTCAGGTACTTC-3′	5′-CGGATTCAGCGAGCTTAGCTT-3′
PDX1.3 qPCR	5′-TTTGCGGTTGCCGGAAT-3′	5′-ATCATCGCCGCACCTTCA-3′
PDX2 qPCR	5′-GTTCATACGTGCTCCAGCTGTT-3′	5′-TTGATGGGACGGGATAATCC-3′
PDX2 (vector)	5′-GGGGACAAGTTTGTACAAAAAAGCAGG CTTCATGACCGTCGGAGTTTTAGC -3′	5′-GGGGACCACTTTGTACAAGAAAGCTGGG TCTTATTGAAATATAGGAAGAT-3′

concentration of plasmid DNA with the appropriate genes. Plasmid DNA containing *PDX1* and the *PDX2* homologues was diluted to 1 ng μl^{-1} and subsequent 1:10 serial dilutions were performed to 1 fg μg^{-1}. All reactions were done in triplicate. Real-time 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 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C 1 min).

4.5. Comparison of quantitative PCR data to published array data

Quantitative PCR data for *PDX1* and *PDX2* was corroborated by published array data using Genevestigator (http://www.genevestigator.ethz.ch/) [41]. Microarray experiments available through Genevestigator were performed in Ulm, Germany using two Affymetrix ATH1 full genome array gene chips for each experiment. Data was obtained from samples collected from green leaf tissue. For these studies (http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenextable2.htm) wild type *A. thaliana* (Col-0) were grown *in vitro* on MS medium. Paraquat stress was induced on 16-day-old plants by the addition of 10 μM methyl viologen to the medium. UV-B stress was induced by exposing 18-day-old plants to UV-B for 15 min.

4.6. Protein complex localization

A. thaliana expressing GFP was kindly provided by Dr. Yang Ju Im and Dr. Wendy Boss (Department of Plant Biology, NC State University). The PDX2–GFP fusion protein was constructed using the gateway system (Invitrogen). *A. thaliana PDX2* was amplified at an annealing temperature of 60 °C (Primers (IDT) listed in Table 1). Amplified products were cloned into the gateway donor vector pDoNR205 and sequenced. The amplified *PDX2* was then cloned into the gateway plant expression vector pK7FWG2 to give an N-terminus GFP fusion. *A. thaliana* plants were transformed by floral dip [11] using *Agrobacterium tumefaciens* strain EHA105. Seeds were plated on MS plates with 100 $\mu g\ ml^{-1}$ kanamycin to select for transformants. Expression in whole plants was verified by western analysis using antibody to GFP (Clontech, Mountain View, CA) in conjunction with a horseradish peroxidase-conjugated secondary antibody and the ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, NJ). Leaf epidermal peels were imaged using a Nikon eclipse E800 fluorescence microscope following fixation of the tissues with 4% formaldehyde. Leaf peels were also plasmolyzed by soaking in 100 mM mannitol solution and imaged without fixation.

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Chapter 3: Pyridoxal kinase SOS4 is required for maintenance of vitamin B_6 – mediated processes in chloroplasts

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ABSTRACT

Vitamin B_6 (pyridoxal 5'-phosphate and its vitamers) is an important cofactor in essential enzymatic reactions involved in amino acid metabolism, synthesis of heme and chlorophyll, synthesis of ethylene, carbohydrate metabolism, and defense against oxidative stresses. In spite of the importance of this vitamin, the regulation of its metabolism is only beginning to be defined. In addition, the consequences of altering vitamin B_6 content on plant growth and development are only beginning to be understood. This study focuses on two different mutants of vitamin B_6-metabolizing enzymes in *Arabidopsis thaliana* that are altered in vitamin B_6 content: a *pdx1.3* mutant in the de novo synthesis pathway that is deficient in vitamin B_6, and a salvage pathway *sos4* mutant that accumulates more vitamin B_6 than wild type plants. We show that despite their differences in B_6 content, both mutants share similar phenotypes, including chlorosis, decreased size, altered chloroplast ultrastructure, root sensitivity to sucrose, and increased expression of key carbohydrate and photosynthetic genes. Although the *sos4* mutant has increased levels of vitamin B_6 in whole leaf tissue, concentrations of B_6 vitamers in chloroplasts were lower in both mutants, due to a significant reduction in the accumulation of phosphorylated vitamers. Our data support the hypothesis that some of the phenotypic consequences shared between the *pdx1.3* and *sos4* mutants are due to B_6 deficiency in chloroplasts, and suggest that SOS4 is required for maintenance of phosphorylated B_6 vitamer

concentrations in chloroplasts. Further, our data are consistent with a diffusion model for transport of vitamin B_6 into chloroplasts.

Other key words:

pyridoxine, pyridoxamine, pyridoxal kinase, pyridoxal phosphate synthase

INTRODUCTION

Vitamin B_6 is a term that is used collectively to refer to six different vitamers: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorylated derivatives pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP). The active form of the vitamin in most organisms is pyridoxal 5' phosphate (PLP) and is synthesized by an enzyme complex made up of two proteins, PDX1 and PDX2 (Ehrenshaft et al., 1999; Osmani et al., 1999; Ehrenshaft and Daub, 2001; Mittenhuber, 2001; Dong et al., 2004; Chen and Xiong, 2005; Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006). The substrates for the synthesis of PLP through this enzyme complex are triose phosphates (glyceraldehyde 3'-phosphate or dihydroxyacetone phosphate), glutamine, and either ribose 5'-phosphate or ribulose 5'-phosphate. This reaction ties vitamin B_6 synthesis with the triose phosphate/pentose phosphate pool, a key component of energy metabolism within the cell. This PLP biosynthesis pathway is found in archaebacteria, most eubacteria, protists, fungi and plants (Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001; Mittenhuber, 2001; Bauer et al., 2004; Belitsky, 2004; Dong et al., 2004; Burns et al., 2005; Chen and Xiong, 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005; Wrenger et al., 2005; Wagner et al., 2006; Tambasco-Studart et al., 2007). *E. coli* and other proteobacteria utilize an alternate biosynthetic pathway, in which two proteins, PDXA and PDXJ, catalyze the synthesis of

pyridoxine 5' -phosphate (PNP) via the condensation of 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose-5-P and nitrogen from glutamate (Hill and Spenser, 1996; Drewke and Leistner, 2001). There is only one report of the de novo pathway in animals in the soybean cyst nematode, *Heterodera glycines* (Craig et al., 2008). In general, however, animals lack the ability to synthesize their own vitamin B_6 de novo and must obtain it from their diets.

In the salvage pathway, the six vitamers of vitamin B_6 are interconverted between each other. In contrast to the presence of two different de novo pathways, the salvage pathway appears to be conserved between all organisms, including animals. Initial characterization of the salvage pathway was done in *E. coli* and animals. The *E. coli pdxH* gene encodes PNP (PMP) oxidase, an FMN-dependent oxidoreductase that converts both PNP and PMP to PLP (McCormick and Merrill, 1980). The *E. coli pdxK* and *pdxY* genes encode kinases; PDXK phosphorylates PN, PM, and PL whereas PDXY phosphorylates PL only (Winkler, 2000). Dephosphorylation of the vitamers by acid and alkaline phosphatases has been of interest in animal studies as only the dephosphorylated forms are imported into cells (McCormick and Chen, 1999; McCormick, 2000). A specific PLP (PNP, PMP) phosphatase has been characterized from human erythrocytes (Fonda, 1992). Finally, a pyridoxal reductase that catalyzes a reversible NADPH-dependent reduction of PL to PN has been characterized in *Schizosaccharomyces pombe* (Nakano et al., 1999;

Morita et al., 2004). Two of the salvage pathway enzymes and their encoding genes have been identified and characterized in plants. Genes encoding a PL (PN,PM) kinase have been cloned from both Arabidopsis and wheat (Lum et al., 2002; Shi and Zhu, 2002; Wang et al., 2004). The PNP (PMP) oxidase has been cloned and characterized from Arabidopsis (Gonzalez et al., 2007; Sang et al., 2007).

PLP is a cofactor for a wide variety of enzymatic reactions catalyzing decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions in amino acid and other metabolic pathways (Drewke and Leistner, 2001). Vitamin B_6 forms a Schiff base construct with the amino group of the substrate (Bender, 1994). In the formation of the Schiff base, vitamin B_6 binds to a lysine in the active site of the enzyme, forming a planar Schiff base intermediate. The vitamin B_6 molecule acts as an electron sink, by which electrons are drawn away from the amino acid. The different enzymes specifically destabilize different bonds in the substrate, resulting in different reactions such as transamination, decarboxylation or racemization. In addition to its role in amino acid metabolism, PLP has been shown to be required in mammalian cells for proper hormone metabolism and transcriptional regulation by interacting with hormone receptors and with a transcriptional co-repressor (Tully et al., 1994; Huq et al., 2007). Different B_6 vitamers are potent antioxidants and may play a role in environmental stress responses (Bilski et al., 2000; Wrenger et al., 2005; Titiz et al., 2006; Denslow et al., 2007).

In plants, PLP is required for many metabolic pathways, including ethylene biosynthesis, chlorophyll biosynthesis, and carbohydrate metabolism. It is a required cofactor for the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosyl –L-methionine (SAM) by the enzyme ACC synthase (ACS), the rate-limiting step in ethylene biosynthesis (Tsang et al., 2003). PLP is an essential cofactor in catalytic reactions for heme and chlorophyll biosynthesis (Vavilin and Vermaas, 2002). In these reactions, vitamin B_6 is required for the ligation of glutamate to $tRNA_{Glu}$ by glutamyl-tRNA synthetase (GTS) and the formation of aminolevulinic acid (ALA) by glutamate-1-semi aldehyde aminotransferase (GSA), the rate-limiting step in chlorophyll biosynthesis. In carbohydrate metabolism, PLP serves as a cofactor for starch and glycogen phosphorylases (Shimomura et al., 1980; Takagi et al., 1981; Fukui et al., 1982). PLP activates ADP-glucose pyrophosphorylase in starch synthesis and inhibits the export of triose phosphates from chloroplasts by the triose phosphate transporter (TPT) (Fliege et al., 1978; Preiss et al., 1991).

We recently identified the gene encoding the PNP (PMP) oxidase (PDX3) in Arabidopsis and characterized the phenotypes, B_6 vitamer levels, stress responses, and expression of pathway genes in *pdx3* mutants as compared to the *sos4* PL (PN,PM) kinase mutant (Gonzalez et al. 2007). This study demonstrated that, contrary to expectations, the *sos4* mutant had elevated levels of total B_6 vitamers, due

to significant increases in PLP levels. Analysis of gene expression data showed that the de novo pathway *PDX1.1* and *PDX1.3* genes and the salvage pathway *PDX3* gene were up-regulated in the *sos4* mutant, suggesting that increased PLP levels were due to increased activity of the de novo pathway as well as increased flux through the salvage pathway.

Here we show that the *sos4* mutant shares a number of phenotypic characteristics with a mutant defective in *PDX1.3*, which encodes one of the three PDX1 isoforms in the Arabidopsis de novo pathway; the others being PDX1.1 and PDX1.2 (Tambasco-Studart et al., 2005). Unlike *sos4*, mutants for *pdx1.3* have decreased levels of B_6 vitamers (Titiz et al., 2006; Wagner et al., 2006). Despite their widely varying levels of vitamin B_6 vitamers and some clear phenotypic differences, the two mutants are both chlorotic, have reduced growth, and show sensitivity of root growth specific to sucrose, altered chloroplast ultrastructure, and altered gene expression. We further show that these phenotypes are correlated with a deficiency in phosphorylated B_6 vitamers in the chloroplast, suggesting that SOS4 is essential for maintenance of adequate levels of phosphorylated vitamers in chloroplasts.

RESULTS

pdx1.3 homozygous T-DNA insertion mutant

The Arabidopsis PDX1.3 gene is 1297 bp long and located on chromosome 5 (At5g01410). It contains has no introns and contains a 930-bp coding sequence. A putative knockout mutant containing a T-DNA insertion in the exon of the gene was identified in the Salk collection, SALK_129277 (Alonso et al., 2003). Plants were screened for presence of the T-DNA by PCR with gene-specific primers and a primer corresponding to the T-DNA sequence (Figure 1). PCR of the T-DNA produced a single 500-bp fragment, compared to an 800-bp fragment amplified from the wild type (WT) gene (Fig. 1). PCR of heterozygous plants amplified one band of each size. Transcription of *PDX1.3* was decreased in the *pdx1.3* mutant (Fig. 2A). Expression of *PDX1.1* (encoding one of the other PDX1 isoforms) and *PDX2* (encoding the glutaminase protein that associates with PDX1) was increased, consistent with regulation of other de novo genes in response to the deficiency of vitamin B_6 caused by the mutation (Fig. 2A).

PDX1 activity in both wild type and the *pdx1.3* and *sos4* mutants was assayed using methods previously developed by Herrero and Daub (Herrero and Daub, 2007). Activity of PDX1 in the *sos4* mutant was increased when compared to wild type activity levels, consistent with values reported by Gonzalez et al (Gonzalez et al.,

2007) (Fig. 2B). Consistent with transcript abundance, enzyme activity of PDX1 in the *pdx1.3* mutant was decreased when compared to wild type plants (Fig. 2B).

Vitamin B_6 levels

B_6 vitamers were extracted from leaf tissue of 6 week-old wild type, *pdx1.1, pdx1.2, pdx1.3, and sos4* mutants and quantified by a yeast bioassay (Fig. 3). The vitamin B_6 content of leaves from *pdx1.1* and *pdx1.2* mutants were not significantly different from wild type levels; this result is consistent with previous results (Titiz et al., 2006). By contrast, the *pdx1.3* mutant contained only 40% of the total B_6 levels found in wild-type plants. This decrease is greater than that reported for the SALK_086418 mutant at this locus (Titiz et al., 2006), but is similar to the vitamin B_6 content of plants mutant for *pdx1.3* due to a point mutation (Wagner et al., 2006). As previously reported (Gonzalez et al., 2007), total vitamin B_6 content of *sos4* plants was significantly higher than in wild type plants, with 150% of the levels assayed in wild type. The major increase in total vitamer levels in *sos4* is due to a large increase in PLP, the active vitamer (Gonzalez et al., 2007).

Phenotypic differences

The *pdx1.3* and *sos4* mutants showed obvious phenotypic differences from wild type (Figure 4A). Although they had significantly different levels of vitamin B_6, both mutants were smaller than normal plants (Fig. 4B). The *pdx1.3* mutant was significantly decreased in size by 39% (+/- 10.2%) when compared to wild type plants. The *sos4* mutant plants were significantly smaller than both wild type and *pdx1.3*, being 47% (+/- 11.2%) smaller when compared to wild type plants .

Both *pdx1.3* and *sos4* mutants were visually chlorotic. Relative chlorophyll content was assayed with a Minolta SPAD-502 Chlorophyll Meter. SPAD values of *pdx1.3* and *sos4* were both significantly lower (ca. 71.1% and 66.7% respectively) when compared to wild type plants (Fig. 5A). Supplementing the plants by watering with 100 μM pyridoxine HCl partially rescued the chlorosis phenotype of the *pdx1.3* mutant, but did not rescue the chlorosis phenotype of *sos4* (Fig. 5A). The recovery of normal phenotype in the *pdx1.3* mutants is consistent with low levels of vitamin B_6 in these plants, while the inability to rescue the chlorosis of the *sos4* mutants is consistent with increased levels of vitamin B_6. Interestingly, when grown under long day conditions, chlorosis of both *pdx1.3* and *sos4* mutants was resolved (Fig. 5B).

Response to osmotic and environmental stress

SOS4 was first identified in a screen for root sensitivity to NaCl (Shi and Zhu, 2002). Gonzalez, et al. (2007) confirmed the dramatic inhibition (> 80% reduction) of root growth of the *sos4* mutant on NaCl-containing medium, although shoot mass was not inhibited by salt. Gonzalez et al. (2007) also showed that *sos4* root growth is significantly inhibited as compared to wild type when grown on MS media supplemented with sucrose, but not mannitol (Gonzalez et al., 2007). We assayed *pdx1.3* seedling root growth as the length of the primary root from plants grown vertically on plates containing MS medium supplemented with 100 mM sucrose, 100 mM NaCl or 100 mM mannitol. Similar to the *sos4* mutant (Gonzalez et al., 2007), growth of *pdx1.3* mutant roots was not significantly inhibited by mannitol when compared to wild type (Fig. 6A). However, in contrast to the *sos4* mutant, growth of the *pdx1.3* mutant plant roots are also not significantly different from wild type roots when grown on medium containing 100 mM NaCl (Fig. 6A). Thus the dramatic salt sensitivity of the *sos4* mutant is not shared by the *pdx1.3* mutant. There was no difference in shoot mass of the *pdx1.3* mutant compared to the wild type plants when grown on media supplemented with NaCl or mannitol (data not shown).

Root growth of both mutants was dramatically inhibited by sucrose (Fig. 7). Root growth of the *pdx1.3* mutant was not significantly different from wild type when grown on MS media without sucrose, whereas *sos4* root growth was significantly

decreased. However, when both *pdx1.3* and *sos4* mutants were grown on medium supplemented with 100 mM sucrose, root growth was almost totally inhibited in contrast to the increase in root growth of wild type seedlings. Supplementation of the medium with PN, PL, or PLP restored normal root growth in the *pdx1.3* mutant, but was unable to reverse root growth inhibition in *sos4* mutants (Table 1). This difference is consistent with the low vitamer levels in the *pdx1.3* mutant. Interestingly, while the root growth of the *pdx1.3* mutant was significantly decreased when seedlings were grown on medium supplemented with 100 mM sucrose, shoot mass did not differ significantly from wild type plants, indicating that the sucrose sensitivity is specific to root tissue (data not shown). The specificity of inhibition of roots and not shoots by sucrose was also reported for *sos4* mutants (Gonzalez et al., 2007). In contrast to the resolution of chlorosis when plants were grown under long day conditions, long days did not restore normal root growth in either *pdx1.3* or *sos4* mutants.

Previous studies with the *sos4* mutant demonstrated that it was significantly more sensitive to chilling stress ($5^{o}C$) than wild type plants, but did not differ in response to high light (1000 $\mu mol\ s^{-1}\ m^{-2)}$ (Gonzalez et al., 2007). We assayed growth of the *pdx1.3* mutant in response to chilling ($5^{o}C$) and high light intensities (1000 $\mu mol\ s^{-1}\ m^{-2}$) (Fig. 6B). As with the *sos4* mutant, the *pdx1.3* mutant did not

significantly differ from wild type in its response to high light. In contrast to the *sos4* mutant, the *pdx1.3* mutant also did not differ from wild type in response to chilling.

Chloroplast ultrastructure

content, we investigated possible differences in chloroplast ultrastructure using transmission electron microscopy (TEM) (Fig. 8). Leaf tissue harvested after 4 hours of light from wild type and mutant plants was fixed, stained and visualized by TEM. Chloroplasts of the both *pdx1.3* and *sos4* showed distinct abnormalities when compared to chloroplasts of wild type plants. Chloroplasts of both mutants are distended and appear to have fewer thylakoid membranes. Areas of dense material associated with crystalline starch were present in both wild type and mutant plants, however mutants also contained areas with low optical density, similar in appearance to phytoglycogen found in isoamylase mutants, *isa1* and *isa2* (Delatte et al., 2005; Streb et al., 2008). We quantified the number of starch granules as well as the areas similar to phytoglycogen on TEM micrographs (Fig. 9). The number of crystalline starch granules did not differ significantly between wild type and either of the two mutants. However, both the *pdx1.3* and *sos4* mutants contained more "phytoglycogen-like" areas than wild type plants .

Starch digestion and quantification

Chloroplast ultrastructure suggested that the *pdx1.3* and *sos4* mutant plants may accumulate phytoglycogen, a highly branched form of starch. Thus, we assayed starch content and relative starch branching from plant tissue harvested after eight hours of light. Starch samples were quantified by extraction and enzymatic digestion with either amylase, amyloglucosidase or with both enzymes, and glucose liberated from these digestions was quantified. Absolute values of glucose liberated following digestion with both enzymes of starch from *pdx1.3* and *sos4* mutants (Fig. 10) did not differ from wild type plants, indicating that total starch content of mutant plants is not different from wild type plants. However, more glucose was liberated by starch isolated from *pdx1.3* mutants than by starch isolated from *sos4* mutants. Relative branching was assayed by comparing glucose released following amyloglucosidase treatment (digests both α-1,4 and α-1,6 bonds) as compared to glucose released following α-amylase treatment (digests α-1,4 only) (Fig. 10). In contrast to expectations based on ultrastructure, but consistent with the similar numbers of starch grains, ratios of glucose content released from amylase and amyloglucosidase digestions did not differ when compared to wild type plants, indicating no difference in starch branching.

Sucrose content

Changes in chloroplasts may also affect accumulation of soluble sugars. Sucrose was extracted from leaf tissue of six week old plants at the end of an 8 hour light period and quantified by GCMS (Fig. 11). Sucrose accumulates to a significantly higher level in leaves of the *pdx1.3* mutant as compared to wild type. Sucrose content of *sos4* plants was intermediate between wild type and the *pdx1.3* mutant, but was not statistically different from either.

Transcript accumulation of carbohydrate metabolism and photosynthetic genes

The altered chlorophyll content, root sensitivity to sucrose and altered chloroplast ultrastructure of both mutants led us to assay expression of genes involved in carbohydrate metabolism and photosynthesis. RNA was extracted from leaves of six-week old soil-grown plants harvested at the end of an eight hour light period, and transcript abundances were assayed by qRT-PCR. The carbohydrate metabolism genes assayed were those encoding the starch debranching enzyme isoamylase 2 (ISA2), the triose phosphate transporter (TPT), starch synthase I (SSI) and ADP-glucose pyrophosphorylase (AGPase). Photosynthetic genes assayed encode chlorophyll *a/*b-binding protein (CAB3) and RuBisCO small subunit (rbcS). Expression of AGPase1 was not changed in either mutant (Figure 12). Transcript

abundances of CAB3, TPT and ISA2 were higher than wild type levels in both mutants. In addition, gene expression of rbcS and SSI are increased in the *sos4* mutant. These results suggest that there is a common basis for causing the metabolic changes in carbohydrate metabolism and photosynthesis in the two mutants. Retrograde signaling from chloroplast to nucleus is well documented in *A. thaliana* (Strand et al., 2003; Nott et al., 2006), and a common chloroplastic environment could explain similarities in gene expression.

Chloroplastic vitamin B_6 content

Several of the shared phenotypes including chlorosis and increased sucrose content are consistent with a deficiency in vitamin B_6 levels. Although the *sos4* mutant has elevated tissue levels of total vitamin B_6 and of PLP, the shared phenotypes suggested that both mutants may have a deficiency of vitamin B_6 in the chloroplast. Therefore, we quantified the content of vitamin B_6 in the chloroplasts of both mutants. Chloroplasts were isolated from 6 week old plants after a prolonged period of darkness (20 hours). Chloroplasts were counted, vitamin B_6 was extracted, and vitamin B_6 content was assayed from volumes of lysed chloroplasts of equivalent number (2×10^8 chloroplasts). We assayed total B_6 vitamers (extracts treated with phosphatase and β-glucosidase), total free (non-glycosylated) vitamers (extracts treated with phosphatase but not with β-glucosidase), and total phosphorylated

vitamers (difference between extracts treated with both enzymes and those treated only with β-glucosidase). Results are shown in Fig. 13. In each of the three lines, there was no significant difference between total vitamer levels and levels of free (non-glycosylated) vitamers, indicating that the vitamers in the chloroplasts are not glycosylated. In contrast to total tissue levels of vitamin B_6, total vitamin B_6 levels in the chloroplasts of the *pdx1.3* and *sos4* mutants are not significantly different from each other. However they are both significantly lower than in wild type plants. This large decrease in total B_6 levels in the *pdx1.3* and *sos4* mutant chloroplasts is due to a deficiency in the phosphorylated vitamers; there was no significant difference between wild type or either of the mutants in levels of the non-phosphorylated vitamers.

Altered kinase expression

SOS4 encodes a PL (PN, PM) kinase. In *E. coli*, an alternate kinase exists that phosphorylates only PL only. We searched the Arabidopsis database for sequences homologous to the *E. coli* PdxY protein. We identified the protein encoded by At5g58730 as having 27% identity/48% similarity to the amino acid sequence of PdxY. We assayed expression of this gene in the wild type and *pdx1.3* and *sos4* mutants (Fig. 12). In both *pdx1.3* and *sos4* mutants, expression of At5g58730 is increased approximately 3-fold in relation to expression levels of wild type plants.

These results are consistent with a lack of PLP within the chloroplast, leading to retrograde signaling and up-regulation of an alternate kinase.

DISCUSSION

Pyridoxal 5'-phosphate (PLP), the active form of the vitamin B_6 vitamer family, is a well-known coenzyme in many cellular reactions, most notably those of amino acid metabolism (Drewke and Leistner, 2001). Vitamin B_6 has been shown to be a potent antioxidant and has been linked to environmental stress responses (Bilski et al., 2000; Chen and Xiong, 2005; Seyithan, 2005; Denslow et al., 2007). In addition, vitamin B_6 has been shown to play a role in carbohydrate metabolism, heme and chlorophyll synthesis, ethylene synthesis, transcriptional regulation and hormone action (Fliege et al., 1978; Shimomura et al., 1980; Takagi et al., 1981; Fukui et al., 1982; Preiss et al., 1991; Tully et al., 1994; Capitani et al., 1999; Vavilin and Vermaas, 2002; Tsang et al., 2003; Huq et al., 2007). In spite of its importance, the impact of altered levels of this vitamin on plant metabolism and phenotype are only beginning to be elucidated.

In this study we identified a new mutant in the de novo biosynthesis pathway at the *pdx1.3* locus and characterized its phenotype in comparison to a mutant in the salvage pathway, *sos4*, because both mutants are significantly altered in levels of vitamin B_6. Several different mutants for the *PDX1.3* locus have been characterized

and shown to be deficient in total vitamin B_6 content as well as in content of the specific vitamers (Titiz et al., 2006; Wagner et al., 2006). By contrast the *sos4* mutant contains significantly higher levels of total vitamin B_6 due primarily to large increases in PLP content (Gonzalez et al., 2007). Our study documents some differences between these two mutants. Most notable was the lack of NaCl sensitivity by the *pdx1.3* mutant. *SOS4* was originally identified as a locus required for salt sensitivity responses (Shi and Zhu, 2002), and our work confirms the extreme salt sensitivity of this mutant. By contrast, root growth of the *pdx1.3* mutant did not differ from that of wild type in the presence of salt. This result contrasts with the results of Titiz et al. (Titiz et al., 2006) and Chen and Xiong (Chen and Xiong, 2005) with a different *pdx1.3* mutant (SALK_086418). Both of these studies identified salt sensitivity in the *pdx1.3* mutant. It is unclear why our mutant does not exhibit salt sensitivity. Both Titiz et al. and Chen and Xiong amend their growth media with 100 mM NaCl. It is possible that our mutant is not salt sensitive, while theirs is. It is unclear whether sucrose was present in their media. If it was, the salt sensitivity seen in their mutants may be due to sucrose content, but attributed to salt. In addition, it has been reported that different types of agar can change morphophysiological responses of Arabidopsis seedlings (Jain et al., 2009). If different types of agar were used, it is possible that this may be responsible for the different response to NaCl stress. In addition to salt stress, mass of whole leaf tissue of the *pdx1.3* mutant did not differ from wild type in

response to chilling stress, whereas our previous study showed significant inhibition of plant growth in *sos4* mutants by chilling (Gonzalez et al., 2007).

Despite having widely different levels of vitamin B_6, most of the phenotypic and metabolic changes identified in the mutants are shared. Both mutants exhibit chlorosis and reduced chlorophyll content, smaller plant mass, sucrose-sensitive root growth, altered chloroplast ultrastructure, and altered expression of genes involved in carbohydrate metabolism and photosynthesis. Some of these phenotypes are consistent with a deficiency of vitamin B_6, a conclusion supported by our supplementation experiments showing that B_6 vitamers can partially rescue the chlorophyll deficiency and completely restore root growth of the *pdx1.3* mutant. Reduced growth and reduced chlorophyll content are consistent with a B_6 deficiency interfering with basic metabolic processes (e. g. amino acid biosynthesis) and with chlorophyll biosynthesis. PLP is a required cofactor for 5-aminolevulinate synthase, one of the key reactions of heme and chlorophyll biosynthesis (Vavilin and Vermaas, 2002). Chlorosis is expected in the *pdx1.3* mutant as a lack of vitamin B_6 would affect synthesis of chlorophyll within the chloroplast. Chloroplast ultrastructure would also be affected by a lack of vitamin B_6, as chlorophyll has been associated with thylakoid membrane structure and function; assembly of the light harvesting complexes and the photosystems is coordinated with chlorophyll synthesis (Vavilin

and Vermaas, 2002). CAB3 gene expression is increased in the *pdx1.3* and *sos4* mutants, perhaps in response to deficiency of chlorophyll within the chloroplast.

Sucrose accumulated to significantly higher levels in the *pdx1.3* mutant with intermediate levels in *sos4*. Vitamin B_6 (PLP) is an essential cofactor for several enzymes localized in the plastid, including AGPase and TPT (Fliege et al., 1978; Morell et al., 1988; Preiss et al., 1991; Ball and Preiss, 1994). While AGPase transcript accumulation was not increased in mutant plants, TPT transcript was increased.

TEM micrographs of chloroplasts of the *pdx1.3* and *sos4* mutants show significant ultrastructural changes including swollen chloroplasts and the presence of areas without crystalline starch. The appearance of the mutant chloroplasts is similar to the chloroplasts of mutants for debranching enzymes (*Atisa1* and *Atisa2*), which have a higher concentration of phytoglycogen, a more highly branched form of starch than amylopectin, a key component of crystalline starch (Delatte et al., 2005). However, extraction and enzymatic digestion of starch did not show differences between the mutants as compared to wild type in starch branching. Overall, indicators of changes in carbohydrate metabolism in the mutants were mixed. Total amounts of starch did not differ between either mutant or wild type. There was no change in transcript accumulation of AGPase, a key starch biosynthesis enzyme that has been shown to be activated by PLP in vitro (Morell et al., 1988; Preiss et al.,

1991; Ball and Preiss, 1994). Sucrose accumulated to significantly higher levels in the *pdx1.3* mutant with intermediate (but not significantly increased) levels in *sos4* as compared to wild type. PLP is known to inhibit transport of triose phosphates out of the chloroplasts (Fliege et al., 1978), and TPT expression was increased in both mutants.

One of the most dramatic of the phenotypes seen in both mutants was an almost total inhibition of root growth in the presence of sucrose. Previous studies of other *pdx1.3* mutants (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006) identified short root growth as a phenotype of this mutant. However, our results did not show any reduction in root growth unless the medium was amended with sucrose. In both the *pdx1.3* and the *sos4* mutants, this sensitivity to sucrose is specific to roots; shoot growth is not altered in comparison to wild type plants. A recent study of root growth in a different *pdx1.3* mutant identified a deficiency in root-synthesized auxin, and suggested that auxin deficiency might be the cause of the short root growth (Chen and Xiong, 2009). However, this study did not address the role of sucrose in this phenotype. Thus, it is not clear if auxin deficiency is linked to sucrose exposure or if this deficiency also occurs when *pdx1.3* mutants are grown without sucrose.

Phenotypic and metabolic changes seen in the mutants led us to test whether both *pdx1.3* and *sos4* mutants were deficient in B_6 levels in the chloroplast, even though overall cellular levels in the *sos4* mutant are high. Our results confirmed this

hypothesis. Chloroplasts of both the *pdx1.3* and *sos4* mutants have a deficiency in total B_6 levels in chloroplasts, due entirely to a deficiency in phosphorylated vitamers. These results indicate that SOS4 is required for maintaining normal levels of phosphorylated vitamers in chloroplasts.

The location of vitamin B_6 de novo biosynthesis in plant cells is not known. Several studies have used GFP fusion proteins to localize the de novo pathway PDX1 and PDX2 proteins in Arabidopsis. Studies have indicated the localization of the GFP-fusion proteins in the cytoplasm (Tambasco-Studart et al., 2005) and in membranes, including the plasma membrane and various endomembranes (Chen and Xiong, 2005; Denslow et al., 2007). Our previous studies with PDX2 (which must form a complex with PDX1 to be active) found that PDX2 was localized primarily at membranes and in the nucleus with some also present in the cytoplasm, but was notably absent in chloroplasts (Denslow et al., 2007), indicating that de novo B_6 biosynthesis may not occur within chloroplasts.

To our knowledge, there are no experimental data on the localization of the known salvage pathway proteins in Arabidopsis. Information on subcellular localization compiled by the SUBA Arabidopsis Subcellular Localization Database (Heazlewood et al., 2007) predicts that SOS for is localized in plastids or mitochondria. There is no indication of cytoplasmic localization for SOS4 based on this database. Sequence predictions for the predicted PL-specific kinase

(At5g58730), which is upregulated in both the *pdx1.3* and *sos4* mutants, also predict localization only in organelles, including nuclei, mitochondria, and plastids. Taken together, our data and the localization results and predictions are consistent with the following hypothesis to explain some of the *sos4* phenotypes. De novo B_6 synthesis occurs in the cytoplasm. Vitamers are transported into the chloroplast in their non-phosphorylated state, because the phosphorylated vitamers do not cross membranes. Non-phosphorylated vitamers are phosphorylated in the chloroplasts by SOS4. The *sos4* mutant has reduced ability to produce PLP within the chloroplast, resulting in a B_6 deficiency within this cellular compartment. The presence of some phosphorylated vitamers is hypothesized to be due to the presence of the kinase encoded by At5g58730, a predicted PL kinase which is predicted to be located in organelles. Transcript accumulation of this gene is increased in both mutants.

Our data are consistent with one of two models for transport of vitamin B_6 into chloroplasts. Two models of vitamin B_6 transport exist: the active transport model and the facilitated transport model. In the active transport model, vitamin B_6 is transported across cellular membranes by a transport protein such as Tpn1p in *S. cerevisiae*, Bsu1p in *S. pombe*, and the ECF family of transporters in prokaryotes (Stolz and Vielreicher, 2003; Stolz et al., 2005; Rodionov et al., 2009). To date, no vitamin B_6 transporters have been identified in *A. thaliana* or any other higher order eukaryotes. In the facilitated diffusion model, non-phosphorylated vitamers diffuse

across cellular membranes and are "trapped" by phosphorylation by pyridoxal kinase, creating a sink for continued diffusion of more non-phosphorylated vitamers into the organelle (Mulligan and Snell, 1976, 1977). Our data are consisted with the facilitated diffusion model. Under this model, we would predict that wild type and *sos4* plants would have identical non-phosphorylated vitamers (as was seen in our experiment). Due to the inability of the *sos4* mutant to phosphorylate non-phosphorylated vitamers within the chloroplast, the "molecular trapping" mechanism is disabled, resulting in a decrease of phosphorylated vitamers within the chloroplast.

In summary we have shown that Arabidopsis mutants deficient for the vitamin B_6 de novo and salvage pathway enzymes PDX1.3 and SOS4 have significantly different levels of vitamin B_6 vitamers, but share a slate of common phenotypes. The common phenotypes are due at least in part to a deficiency in phosphorylated B_6 vitamers in chloroplasts, documenting an essential role for the SOS4 kinase in maintaining normal vitamin B_6 metabolism in the chloroplast.

METHODS

Strains and growth conditions. This study utilized wild type Arabidopsis ecotype Col-0. The *sos4* mutant, with a point mutation in the locus encoding the PL(PN,PM) kinase (Shi and Zhu, 2002) was kindly provided by Dr. Jian-Kang Zhu, UC-

Riverside. Recovery of a homozygous *pdx1.3* mutant line is described below. For vitamin B_6 analysis, T-DNA insertion lines with inserts in the two additional PDX1 homologs were obtained from the ABRC. The *pdx1.1* mutant is GABI_612B04 which has a T-DNA insertion at the 3' untranslated region. The *pdx1.2* mutant is SALK_148199, which has a T-DNA insertion in the exon.

Seeds were surface sterilized by incubation for 5 minutes with 70% ethanol, followed by three rinses with sterile dH_20. Seeds were then incubated in a 50% bleach 0.1% Triton X-100 solution and subsequently rinsed four times with sterile dH_20. Seeds were suspended in 0.1% agarose (top agar) and plated on Murashige and Skoog (MS) salts medium supplemented with Gamborg's vitamins. Plates were wrapped in aluminum foil and stratified at 4˚C for 48 hours. Plates were then moved to a 22˚C incubator for 48 hours. After 48 hours, the plates were unwrapped and incubated for one week under short day conditions (8 hours light/16 hours dark). Seedlings were then transferred to either soil or appropriate agar medium.

Soil-grown plants were also grown from seed sown directly onto soil. Seeds which had been stored at 4˚C were suspended in dH_20 and then pipetted directly onto soil (PM-15-13 AIS mix [Lehle Seeds]. Flats of plants were covered with plastic domes for two days and seeds were allowed to germinate. After germination, lids were removed. Plants were thinned to one plant per pot between 1 and 2 weeks after

germination. Plants were watered once a week and grown under laboratory conditions (20-22˚C, 8 hour photoperiod, 80 μmol s^{-1} m^{-2} light).

Plants for chilling and high light experiments were germinated on MS agar as described above and transferred to soil at one week. Plants were grown in growth chambers under control conditions (20˚C, 8 hours light, 200 μmol s^{-1} m^{-2} light) for three weeks. After three weeks, one third of the plants were moved to high light conditions (20˚C, 8 hours light, 1000 μmol s^{-1} m^{-2} light) and one third of the plants were moved to chilling conditions (5˚C, 8 hours light, 200 μmol s^{-1} m^{-2} light). Plants were grown under experimental conditions for two weeks, at which time all plants were harvested for further analysis (Gonzalez et al., 2007).

Recovery of Homozygous *pdx1.3* T-DNA Insertion Mutant Line

Plants from the Arabidopsis ecotype Col-0 with a T-DNA insertion located in the coding region of the pdx1.3 gene (SALK_129277) were obtained from the ABRC (Alonso et al., 2003). These mutants were screened by PCR to verify the insertion and isolate homozygous mutant plants. Seeds were surface sterilized and germinated on medium as described above. Seedlings were transferred to Arabidopsis growing medium PM-15-13 AIS mix (Lehle Seeds) and grown under an 8 hour photoperiod for three weeks. DNA was extracted from leaves using the Quick DNA Prep for PCR protocol (Weigel and Glazebrook, 2002). The T-DNA insertion was screened by a

three primer screening strategy. *PDX1.3* primers (forward primer, 5'-CAACACCGCCACATGCCATT-3'; and reverse primer 5'-ATAGAAGTTCTCGCCACTGG-3') were designed using the SIGnal T-DNA verification primer design tool, powered by the Genome Express Browser Server (http://signal.salk.edu/tdnaprimers.2.html). In addition, a left border primer of the T-DNA insertion (Lba1, 5'-TGGTTCACGTAGTGGGCCATCG-3') was also added to the reaction. Amplification was achieved under the following conditions: 95˚C for 5 mins, followed by 40 cycles of 95˚C for 1 min, 56˚C for 1 min, 72˚C for one min and 72˚C for seven mins.

Vitamin B_6 extraction

Vitamin B_6 extraction methods were adapted from Denslow et al. and Gonzalez, et al. (Denslow et al., 2005; Gonzalez et al., 2007). Fresh leaf issue (0.5 g) was ground with a mortar and pestle and autoclaved with 30 ml 0.44 N HCl for four hours. The pH of the extract was adjusted to 4.8 with sodium acetate, followed by treatment overnight (37˚C with shaking at 70 RPM) with freshly prepared β-glucosidase and acid phosphatase (β-Glucosidase, Sigma (St. Louis, MO), 2.1 units/mg; Acid Phosphatase, Sigma (St. Louis, MO), 0.4 units/mg). These treatments convert the glycosylated and phosphorylated vitamers to free vitamers for uptake by the yeast. Samples were filtered through miracloth to remove cellular debris, brought

to 35 ml with dH_20 and refiltered with a Steriflip filtering device (0.22 μM; Millipore) to sterilize the sample. Samples were wrapped in aluminum foil to protect the samples from light and stored at -20˚C until use in the auxotrophic yeast assay.

Vitamin B_6 was assayed using a protocol modified by Denslow et al. (Denslow et al., 2005). To assay B_6 content, vitamin B_6-auxotrophic yeast cells (ATCC 9080) were grown on malt agar medium overnight at 30˚C. Cells were collected from the agar plates by washing three times with 10 ml of sterile, deionized water and pelleted by centrifuging at 1,000g for ten minutes (4˚C). Pellets were resuspended in 5 ml of sterile deionized water, and the concentration of the cell suspension was determined using a hematocytometer. The vitamin B_6 extracts described above were added (50μl) to 4 ml of PyrY liquid medium (Beckton Dickson) and the mixture inoculated with $5x10^6$ yeast cells. Standards contained 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 ng of total PN per 4 ml volume. All samples and standards were run in triplicate and incubated overnight with shaking (30˚C, 250 rpm, dark). Yeast growth was quantified by measuring absorbance at OD_{540} nm at 16 and 24 hours post inoculation using either a Beckmann DU 650 spectrophotometer or a Nanodrop ND-1000 Spectrophotometer. Total B_6 vitamer content of the extracts was quantified by comparison to the standard curve.

PDX1 enzyme assay

Leaf tissue samples from both frozen tissue of adult plants (-80˚C) and three week old seedlings were treated protein activity was assayed as previously described by Herrero et al. (Herrero and Daub, 2007). Two samples each of each genotype was harvested and processed for each biological replicate.

Relative chlorophyll content determination

Relative chlorophyll content was assayed by measuring chlorophyll fluorescence using a Minolta SPAD-502 Chlorophyll Meter (Richardson et al., 2002). Measurements were taken from six week old plants grown in soil after four hours of light. Two measurements were made from separate mature leaves of each plant. Results are the average of at least two separate biological replicates of at least eight plants each.

Root assay

Seedlings of the *pdx1.3* and *sos4* mutant lines and the wild type were grown as described above. After one week, seedlings were transferred to plates of MS medium supplemented with 100 mM sucrose, 100 mM NaCl or 100 mM mannitol. Plates were stored vertically under short day (8 hours) conditions or long day (16

hours) conditions and root growth was scored every two or three days for two weeks. Data shown are the results of one experiment. Unless otherwise noted, the experiment was repeated at least three times with similar results.

Electron microscopy

Samples for electron microscopy were prepared according to the methods of Dykstra and Reuss (Dykstra and Reuss, 2003). Mature leaves of six week old Arabidopsis plants were harvested after 4 hours light (8 hour daylength, 80 μM light) and immediately submerged in 4F:1G fixative under vacuum and stored at 4˚C until preparation for embedding. Samples were rinsed in 0.1 M Sorenson's sodium phosphate buffer (pH 7.2-7.4) and post-fixed in 1% osmium tetroxide/0.1M phosphate buffer. Tissue was rinsed with distilled water and dehydrated by passing through an ethanol series, followed by two rinses in 100% acetone. Samples were then embedded in Spurr resin and polymerized overnight at 70 ˚C. Ultrathin sections (70-90 nm) were cut with a glass knife, collected on copper grids and stained with methanolic uranyl acetate followed by lead citrate. Ultrathin sections were examined with a FEI/Philips EM 208S Transmission Electron Microscope.

Starch isolation and digestion

Starch was extracted using methods modified from Winter, et al. (Winter, 1993). Fresh leaf tissue (six weeks of age, 8 hours light/16 hours dark, harvested after 8 hours of light) was frozen in liquid nitrogen immediately after harvest. Soluble sugar was extracted as described below. Following soluble sugar extraction, the organic fraction was brought to 10 ml with 95% ethanol and cellular debris was pelleted by centrifugation. The pellet was subsequently resuspended in 2 ml of 0.2 N KOH, heated at 70˚C for 4 hours in a shaking water bath and cooled to room temperature. pH was adjusted to 4.5-5.0 with acetic acid and sonicated in a sonication water bath for 10 minutes.

100 µl of resulting starch sample was combined with 400 µl of starch digestion buffer (50 mM sodium acetate (pH 4.8) containing either 33 nkat of amylase or 23 nkat of amyloglucosidase, or appropriate concentrations of both enzymes) and incubated for 16 hours at 37˚C. Insoluble debris was pelleted by centrifugation, and 50 µl of the clarified supernatant was assayed for glucose content through enzymatic assay.

Glucose content was determined by monitoring the formation of NADPH in a 1 ml reaction containing buffer (50 mM imidazole, 1.5 mM $MgCl_2$, pH 6.9), 50 µM NADP, 100µM ATP and 50 µl of soluble sugar extract. The reaction progress was monitored spectrophotometrically at 334 nm (405 nm background wavelength). 100

nkat each of glucose-6-phosphate dehydrogenase and hexokinase, were added sequentially, allowing the absorbance to flatline before adding the next enzyme. Changes in absorbance were compared to absorbances of a range of glucose standards of known concentrations.

Sucrose analysis

Sucrose was extracted using methods modified from Winter, et al. (Winter, 1993). Fresh leaf tissue was frozen in liquid nitrogen immediately after harvest. Tissue was thoroughly ground in liquid nitrogen for twenty minutes followed by an additional twenty minutes of grinding in 5.6 ml extraction buffer [3.5 ml methanol, 1.5 ml chlorophyll, 0.6 ml phosphate buffer (20 mM KH2PO4 (pH 7.0), 5 mM EGTA, 20 mM NaF)]. Sucrose was extracted three times with water, dried, resuspended and stored at -20 ˚C. Sucrose content was quantified by GCMS.

GCMS analysis was performed according to the methods of Glassbrook (Glassbrook, 1999). Extracts were mixed with acetonitrile and reduced to dryness under vacuum. Dried extracts were resuspended in 400 µL of derivatization solvent (acetonitrile containing 100 mM N-methylmorpholine and 50 mM trifluoracetic acid with 3 ug/mL octyl-β-D-glucopyranoside as an internal standard), followed by mixing with 100 µL of derivatizing reagent (90% v/v N,N-dimethyltrimethylsilylamine and 10% v/v hexamethyldisilazane) and incubation at 50°C for 1 hour to convert sugars to

trimethylsilyl (TMS) derivatives. The resulting extracts were centrifuged and the supernatant was analyzed by GC-MS.

GCMS was performed on a Thermo Trace GC Ultra gas chromatograph/Thermo DSQII mass spectrometer equipped with an electron impact ion source with a Restek Rtx®5Sil MS (5% phenyl polysiloxane) column (30 m; 0.25 mm I.D.; 0.25 µm film thickness). The carrier gas was helium set at a constant flow of 1 mL/min (linear velocity of ~40 cm/s). One microliter of extract was injected into a PTV injector operated in splitless mode at 160°C, raised immediately at 5 °C/s to 360°C, held at 360°C for 3 min, lowered at 2 °C/s to 240°C, then held at 240°C. The column oven temperature was programmed for a 60°C initial temperature with an 8 °C/min ramp to a final temperature of 360°C. The mass spectrometer was operated with an electron impact source in positive mode monitoring m/z 191, 204, 217, 361, 437.

Data were quantified by comparing peak areas obtained for TMS-derivatives in the sample with reference standards analyzed concurrently with the extracts. The chromatographic data were processed using Xcalibur® 1.4 software (Thermo, Waltham, MA).

RNA extraction and quantitative Real Time PCR

Total RNA was extracted from six week old leaf tissue after eight hours of light using the Promega SV Total RNA Isolation System. One µg of RNA per sample was reverse transcribed as previously described (Denslow et al., 2007). Gene expression of selected genes was determined in wild type plants in addition to *pdx1.3* and *sos4* mutants. One µl of cDNA was added to 12.5 µl 2x SYBR Green Master Mix (SensiMixSYBR®, Quantace, London, UK) and gene-specific primers (0.4 µM). Reactions were brought to 25µl with sterile deionized water. All primers were designed with the Primetime qRT-PCR Assay tool (http://www.idtdna.com/Scitools/Applications/RealTimePCR/) except for the primer sequences for PDX2 gene and the PDX1 homologs, which were obtained from Gonzalez, et al. (Gonzalez et al., 2007) (Table 2). cDNA sequences were obtained from NCBI. Transcript abundances of all genes were compared to the levels of Actin 1 (At2g37620) mRNA. Reactions were run in triplicate on a DNA Engine Opticon 2 553 System (Bio-Rad Laboratories, Hercules, CA) using the following program: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15s and 60˚C for 1 minute. The experiments were repeated at least two times from RNA isolated from at least two separate biological replicates.

Chloroplast isolation and vitamin B_6 quantification of chloroplast fraction

Arabidopsis chloroplasts were isolated from fresh leaf tissue using the Chloroplast Isolation Kit (Sigma, St. Louis, MO). Ten grams of fresh leaf tissue was pulsed 4 times for 2 seconds each in a blender with 45 ml of Chloroplast Isolation Buffer with 0.1% BSA supplied by the manufacturer followed by filtration through a nylon filter. Chloroplasts were pelleted by centrifugation and the pellet was gently resuspended in 1 ml of chloroplast isolation buffer with 0.1% BSA added. The supernatant was discarded. Chloroplasts were further separated from other cellular components by centrifugation in a 40% Percoll solution and then resuspended in 2 ml of chloroplast isolation buffer without BSA. Chloroplasts were counted with a hematocytometer. After counting, 500 µl lysis buffer (62.5 mM Tris HCl, pH 7.5; 2 mM $MgCl_2$) was added to 500 µl chloroplast suspension. Samples were treated with either 100 µl acid phosphatase (5 mg/ml), 100 µl β-glucosidase (37.5 mg/ml), or 100 µl each of acid phosphatase and β-glucosidase. Samples were incubated overnight at 37˚C with mild shaking. Samples were centrifuged to pellet debris, and the supernatant was collected for each sample. Supernatant was brought to two ml with sterile dH_2O and filter sterilized using Corning™ 0.2 µm sterile syringe filters.

Volumes equivalent to 2 x 10^8 chloroplasts of each sample were added to 4 ml of PyrY liquid medium and assayed for B_6 content using the yeast bioassay described above. Results were calculated for total B_6 vitamers (extracts treated with both β-

glucosidase and acid phosphatase), total free (non-glycosylated) vitamers (treated with β-glucosidase only), and total non-phosphorylated vitamers (treated with acid phosphatase only). Total phosphorylated vitamer levels were calculated as the difference between extracts treated with both enzymes and with acid phosphatase only. Results reported are from two separate biological replicates.

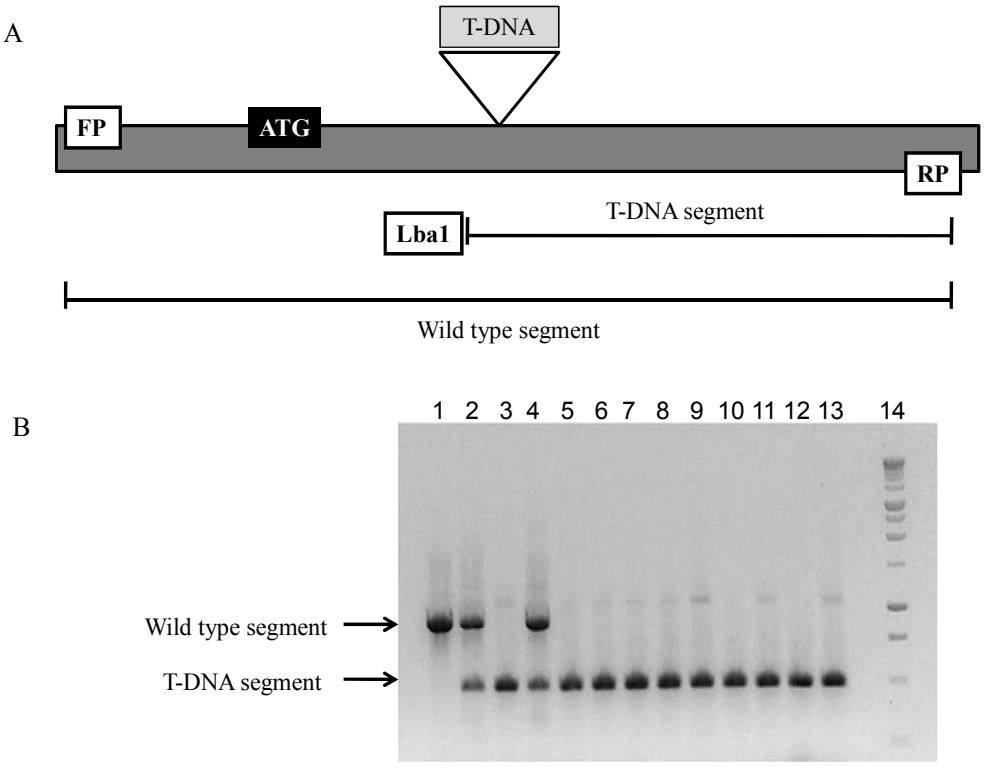


Figure 1. **Isolation of the *pdx1.3* insertion mutant.** A. Location of the T-DNA insertion in the *pdx1.3* mutant line (SALK _129277) within the *PDX1.3* gene. Gene specific forward (FP) and reverse primers (RP) amplified the 800 bp wild-type *PDX1.3* segment. Lba1 is the T-DNA left-border primer used with RP to amplify the approximately 500 bp segment in the T-DNA insertion mutants. B. Screening of T-DNA *pdx1.3* insertion lines by PCR. DNA from plants segregating for the T-DNA insertion was amplified with the FP, RP and Lba1 primers to amplify the 800 bp wild type fragment and/or the 500 bp T-DNA insertion fragment. Wild type plants, lacking the T-DNA insertion, amplified only the 800 bp fragment, while homozygous mutant plants amplified only the 500 bp fragment. *pdx1.3* heterozygous plants amplified both the 800 bp and 500 bp fragments. Lane 1: wild type; lanes 2, 4, *pdx1.3* heterozygous plants; lanes 3, 6-14 *pdx1.3* homozygous mutants.

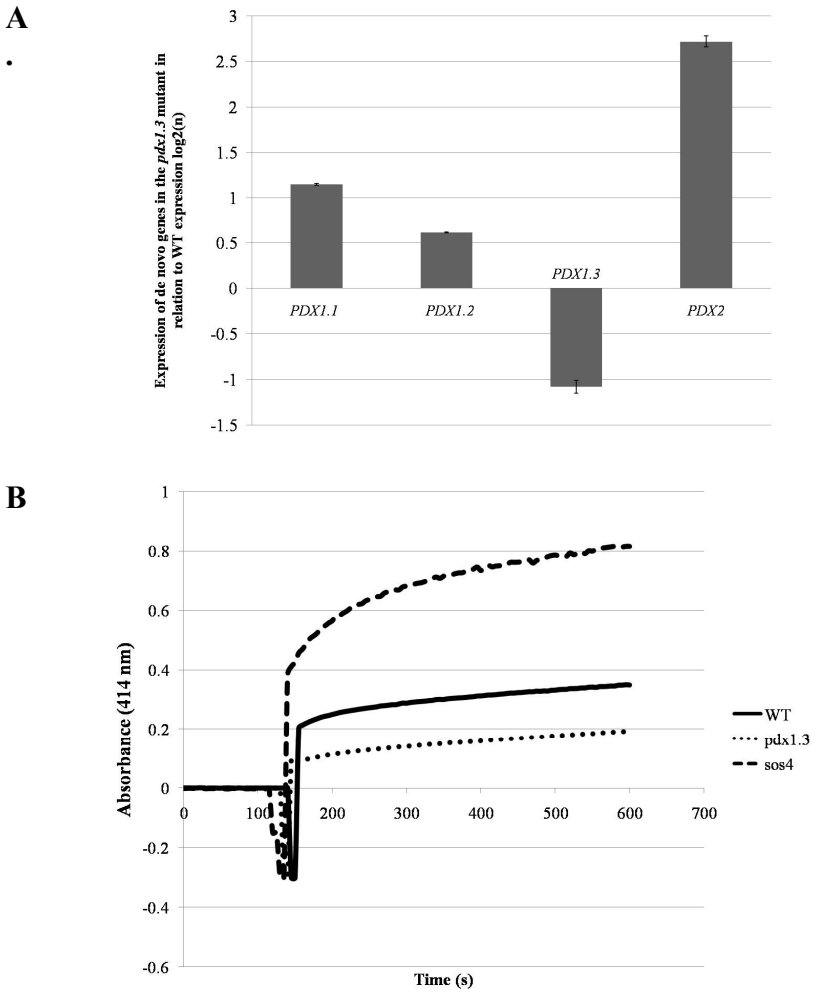


Figure 2. De novo pathway gene expression of *pdx1.3* insertion mutants and PDX1 enzyme activity in *pdx1.3* and *sos4* mutants. A. Expression of vitamin B_6 de novo biosynthesis genes in the *pdx1.3* T-DNA insertion mutant (SALK_129277) assayed by qRT-PCR. Data represents log2(n) of mutant transcript abundance over wild type levels. Data represent one biological replicate with three technical replicates. Two biological replicates were performed with similar results. Error bars represent standard error. **B.** Enzyme activity of PDX1 in wild type and *pdx1.3* and *sos4* mutants. Substrate added at 100 s. Data are represented as absorbance. Data represent one biological replicate with two technical replicates.

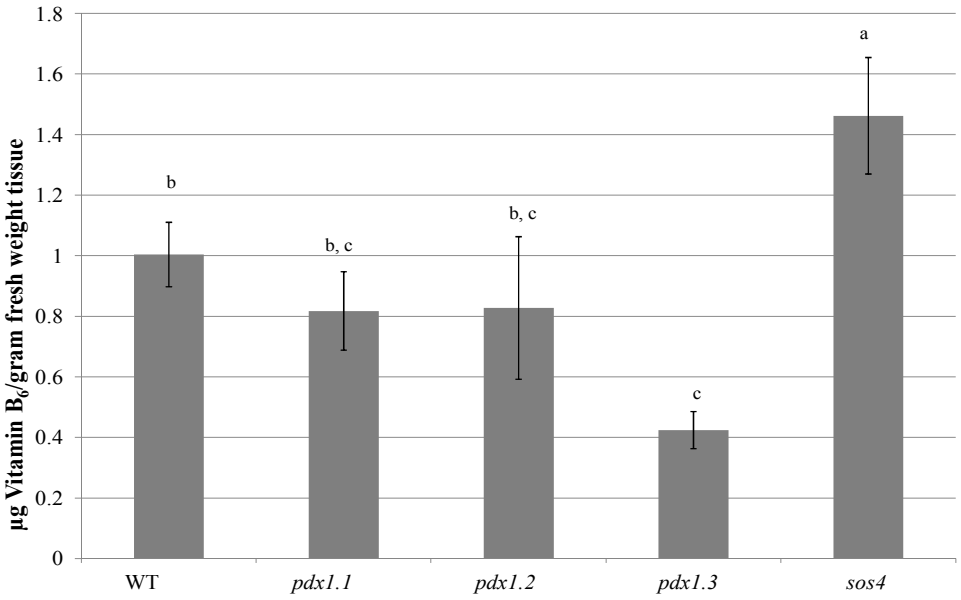


Figure 3. **Vitamin B_6 content is decreased in *pdx1.3* mutants, while increased in *sos4* mutants**. Vitamin B_6 content was determined by auxotrophic yeast bioassay of extracts treated with β-glucosidase and acid phosphatase to convert glycosylated and phosphorylated vitamers to free vitamers for uptake by the yeast. Total vitamin B_6 values represent the average concentration in plant extracts obtained from at least two independent sets of plants. Means followed by separate letters are significantly different at $P = 0.05$ according to Duncan's Multiple Range Test. Error bars represent standard error.

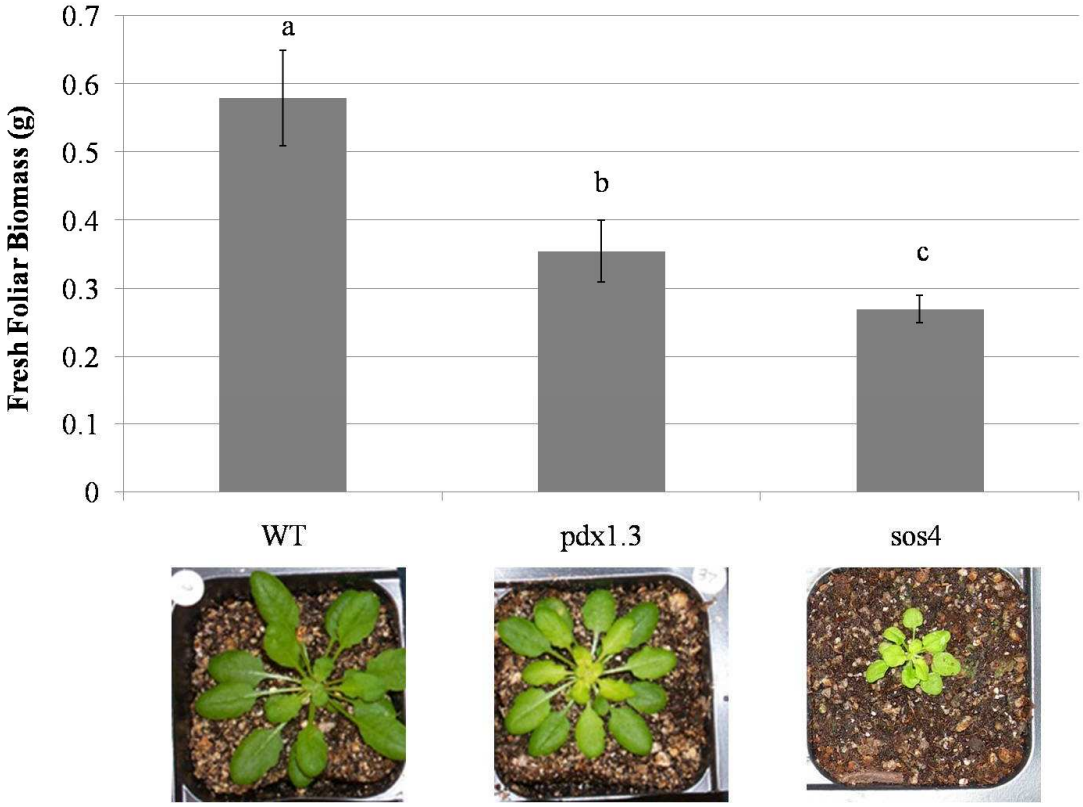
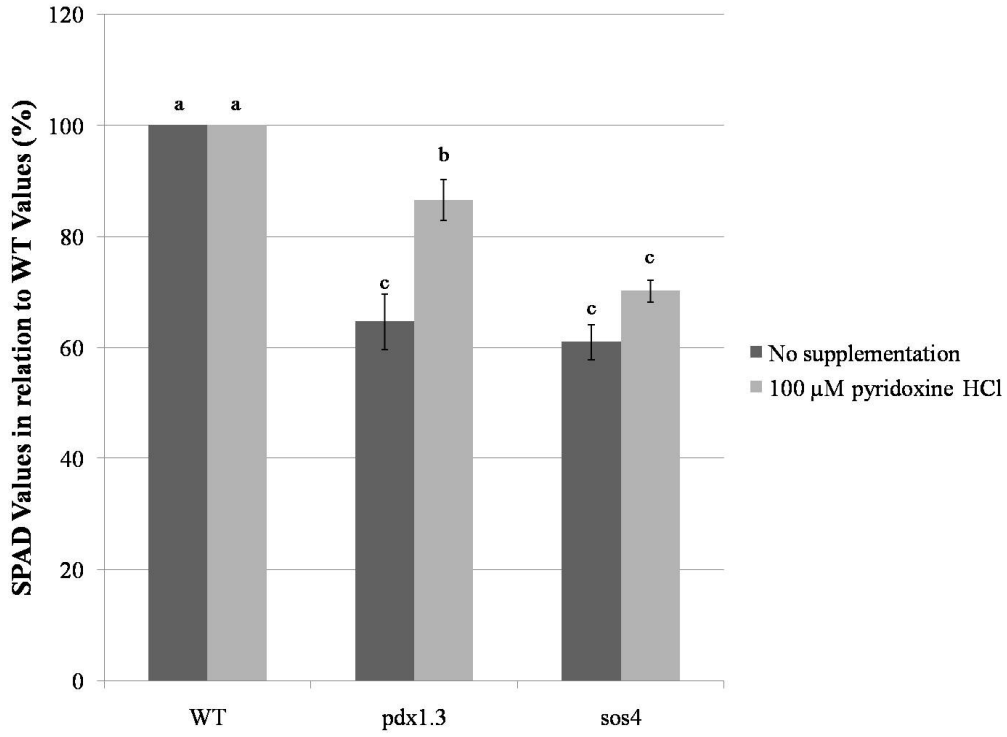


Figure 4. Both *pdx1.3* and *sos4* mutants are smaller than WT plants. Plant mass averages are the result of at least two independent biological replicates. Seeds were sown in soil under controlled conditions (20-22˚C, 80$\mu m^{-1}s^{-2}$, 8 hours light). Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

Figure 5. **Chlorophyll levels of *pdx1.3* and *sos4* mutants are decreased in relation to WT plants. A.** Chlorophyll levels in wild type, *pdx1.3*, and *sos4* mutants, with and without pyridoxine supplementation, were assayed through measurement of chlorophyll fluorescence (SPAD). SPAD values are represented in relation to wild type SPAD values (%). Both *pdx*1.3 and *sos4* mutant plants have significantly lower SPAD values than wild type plants. Chlorosis of the *pdx1.3* mutant, but not the *sos4* mutant is partially rescued by supplementation with 100 μM pyridoxine. **B.** Chlorophyll levels of WT and both *pdx1.3* and *sos4* mutants increase under long day conditions. SPAD values of *pdx1.3* and *sos4* mutants grown under long day conditions exceed SPAD values of WT plants grown under short day conditions. Therefore, long day conditions relieve the chlorotic phenotype of both *pdx1.3* and *sos4* mutants grown under short days. SPAD values were measured with a Minolta SPAD-502 chlorophyll meter and are the average of at least two independent biological replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

A.


SPAD Values in relation to WT Values (%)
120
100
80
60
40
20
0
a
a
b
c
c
c
WT
pdx1.3
sos4
No supplementation
100 µM pyridoxine HCl

B.

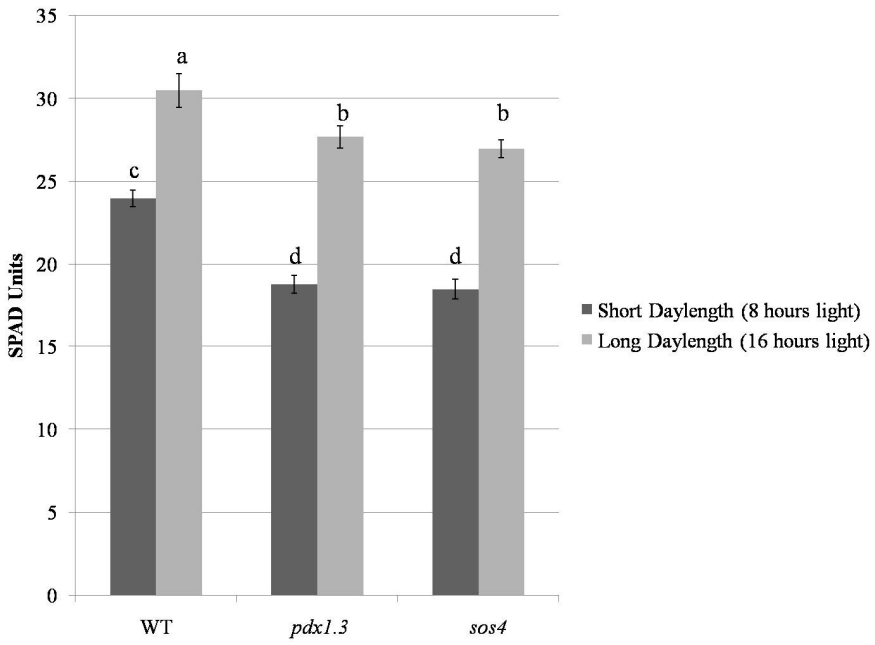
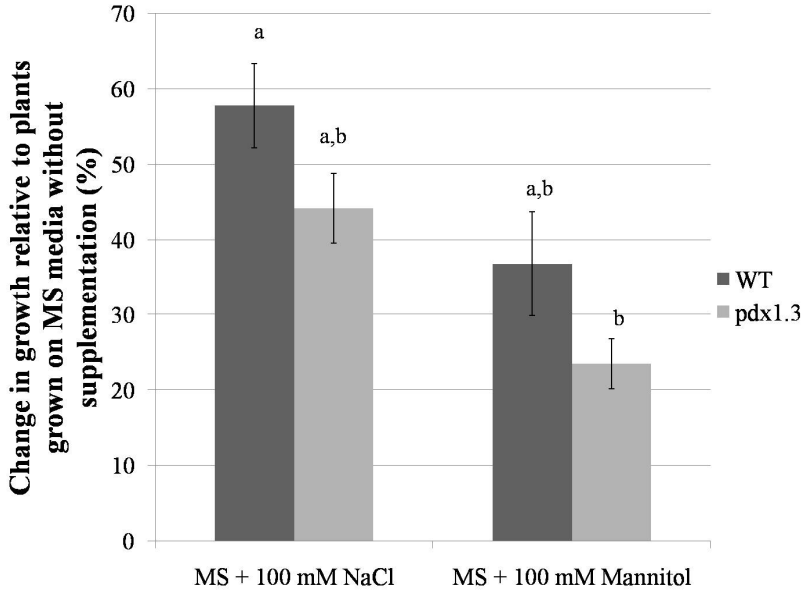
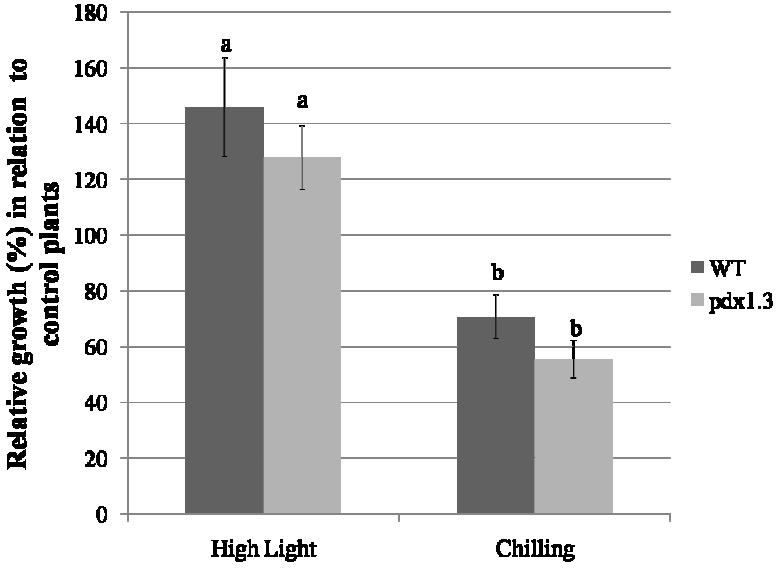

SPAD Units
35
30
25
20
15
10
5
0
a
b
b
c
d
d
WT
pdx1.3
sos4
Short Daylength (8 hours light)
Long Daylength (16 hours light)

Figure 6. Response of the *pdx1.3* mutant to osmotic and environmental stress. **A.** Root growth of *Arabidopsis thaliana* Col-0 (wild type) and *pdx1.3* mutant when grown on MS media supplemented with 100 mM NaCl or mannitol. Root growth of the *pdx1.3* mutant does not differ from wild type when grown on MS medium containing salt or mannitol. Root growth averages represent measurements of root growth taken after ten days on experimental media and the result of at least three independent biological replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error. **B.** Response of wild type and *pdx1.3* mutant plant grown under high light and chilling conditions. Relative dry weight of plants in high light (20˚C, 8 hours light, 1000 µmol $s^{-1}m^{-2}$ light) and chilling (5˚C, 8 hours light, 200 µmol $s^{-1}m^{-2}$ light) conditions. Measurements are the result of two independent biological replicates. Means followed by the same letter are not statistically significant at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

A.


Change in growth relative to plants grown on MS media without supplementation (%)
70
60
50
40
30
20
10
0
a
a,b
a,b
b
WT
pdx1.3
MS + 100 mM NaCl
MS + 100 mM Mannitol

B.


Relative growth (%) in relation to control plants
180
160
140
120
100
80
60
40
20
0
a
a
b
b
WT
pdx1.3
High Light
Chilling

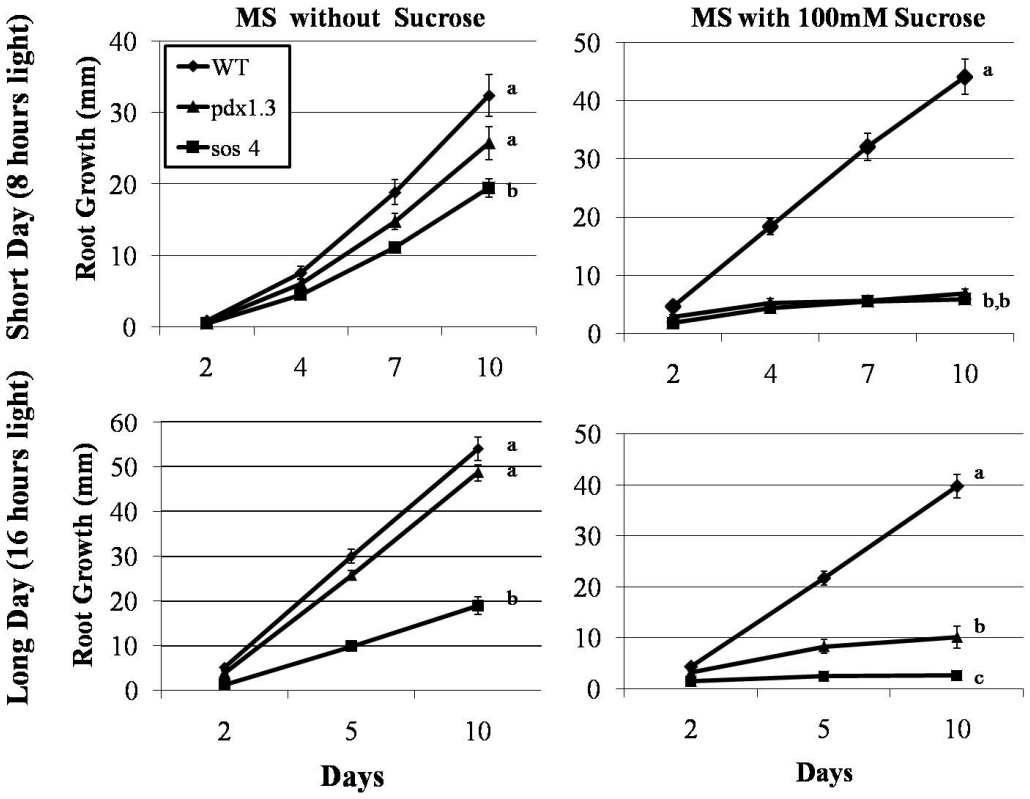


Figure 7. Root growth of the *pdx1.3* and *sos4* mutants grown on MS tissue culture medium with and without 100 mM sucrose supplementation under short and long day conditions. Seeds were germinated on MS medium and transferred to experimental media (MS alone, MS + 100 mM sucrose) after one week. Agar plates were oriented vertically and root growth was measured every two or three days. Data shown represent one biological replicate with at least six plants per group. Experiment was repeated three times under short day conditions and two times under long day conditions with similar results. Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

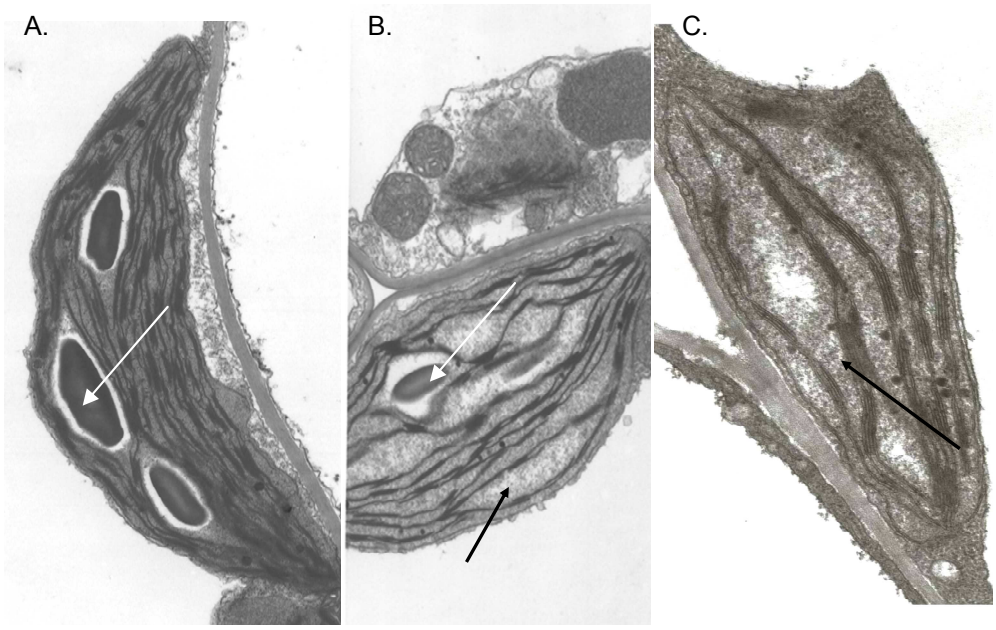


Figure 8. Chloroplast ultrastructure from leaves of wild type, *sos4* and *pdx1.3* mutants. Chloroplasts of both the *pdx1.3* and the *sos4* mutants appear swollen instead of lens shaped, and both mutants have fewer thylakoid membranes and smaller stacks of grana. In addition, starch structure appears to be altered in the mutants. In addition to the presence of multiple starch granules (white arrow), mutant chloroplasts also contain areas filled with less optically dense regions similar to phytoglycogen (highly branched starch). Black arrows indicate areas of the chloroplast which appear to contain more highly branched starch.

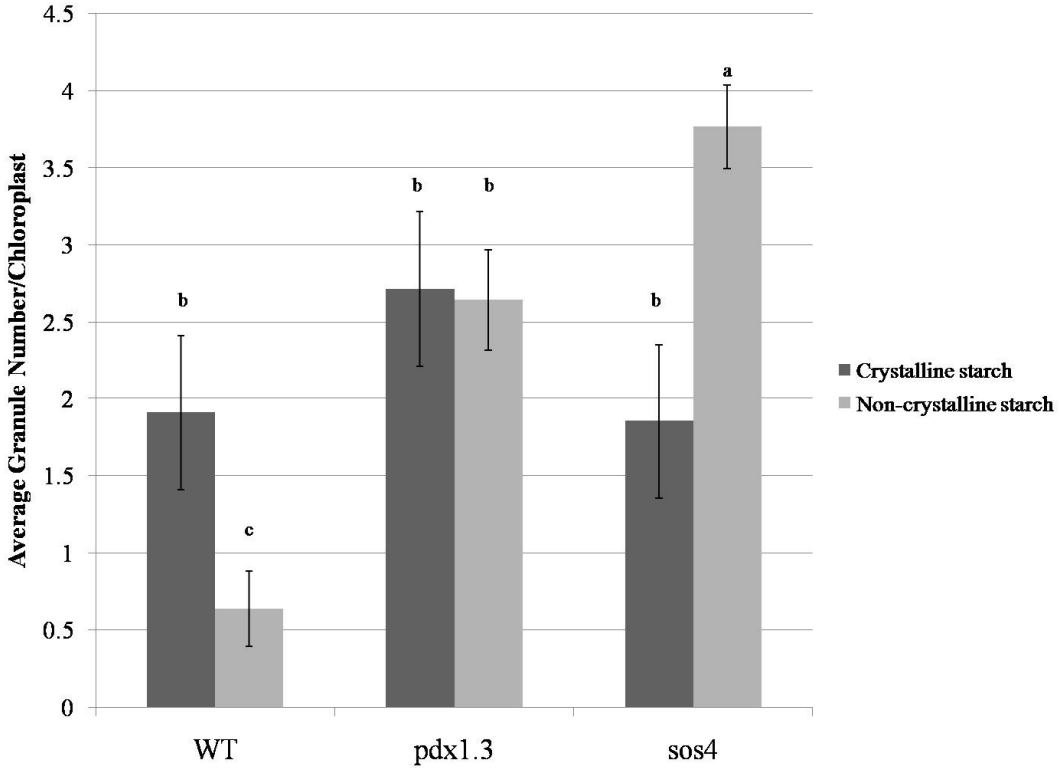


Figure 9. Number of crystalline starch granules and "phytoglycogen-like" areas from TEM micrographs of wild type and *pdx1.3* and *sos4* chloroplasts. Numbers of crystalline starch granules are similar in wild type and *pdx1.3* and *sos4* mutants, however, both mutants contain more "phytoglycogen-like" regions than wild type plants. Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

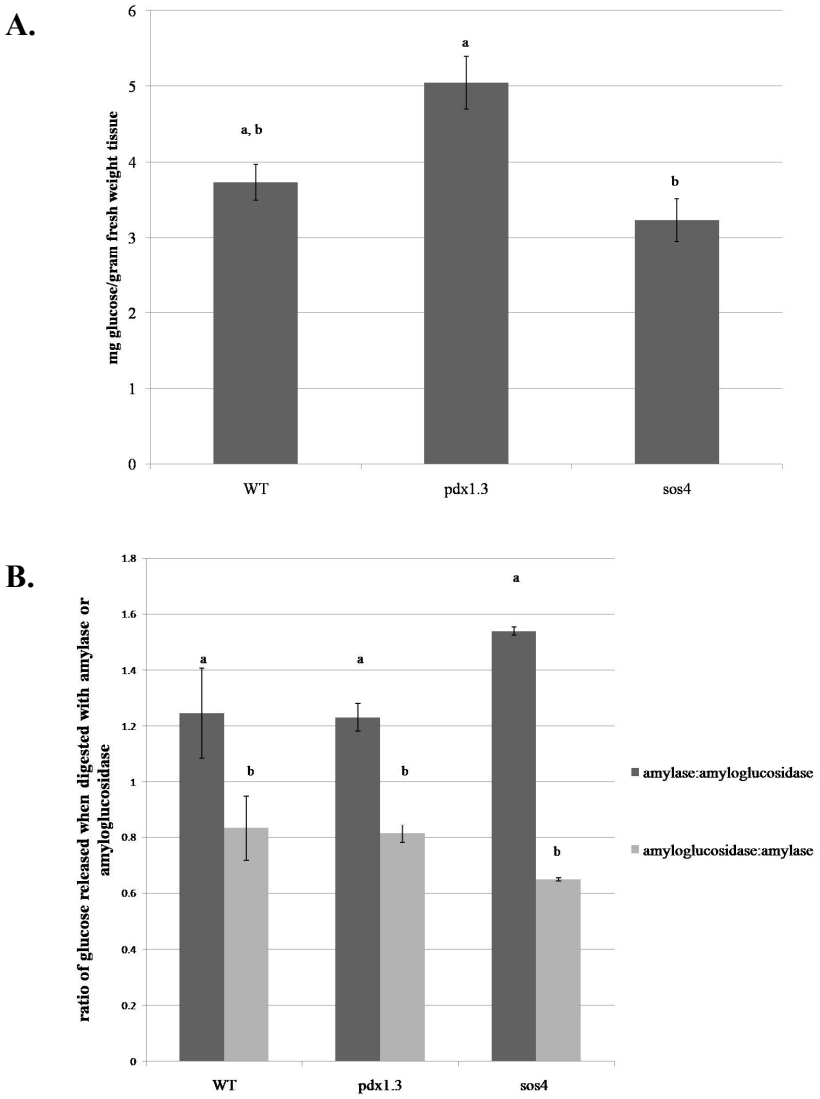


Figure 10. Enzymatic analysis of starch granule composition. A. Total glucose released from enzymatically digested starch. **B.** Ratio of glucose released between amylase and amyloglucosidase digestions. Presence of more branched starch would yield a lower amylase:amyloglucosidase ratio and a higher amyloglucosidase:amylase ratio. However, no significant differences are present between the mutants as compared to wild type. Glucose quantities are the result of two independent biological replicates. Means followed by the same letter are not statistically significant at $P \leq 0.05$ according to Anova analysis. Error bars represent standard error.

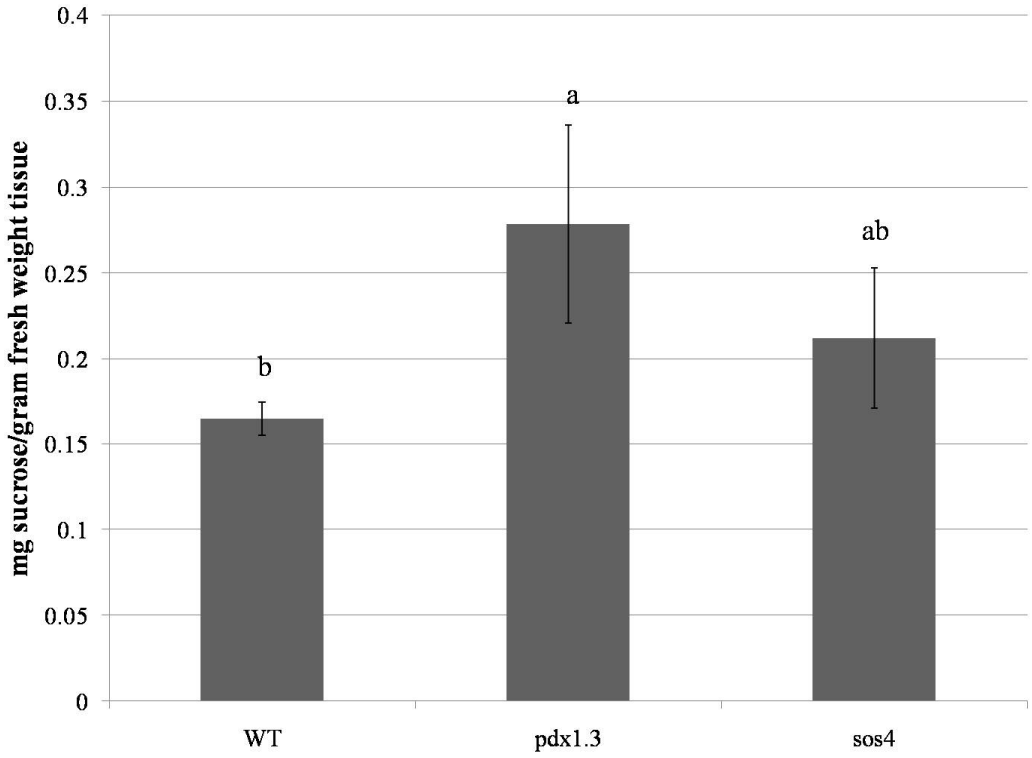


Figure 11. **Sucrose content of wild type and *pdx1.3* and *sos4* mutants.** Sucrose was extracted from six week old plants harvested after eight hours of light. Sucrose content was quantified by GCMS and data shown are the result of two independent biological replicates. Means followed by the same letter are not statistically significant at $P \leq 0.05$ according to Duncan's multiple range test. Error bars represent standard error.

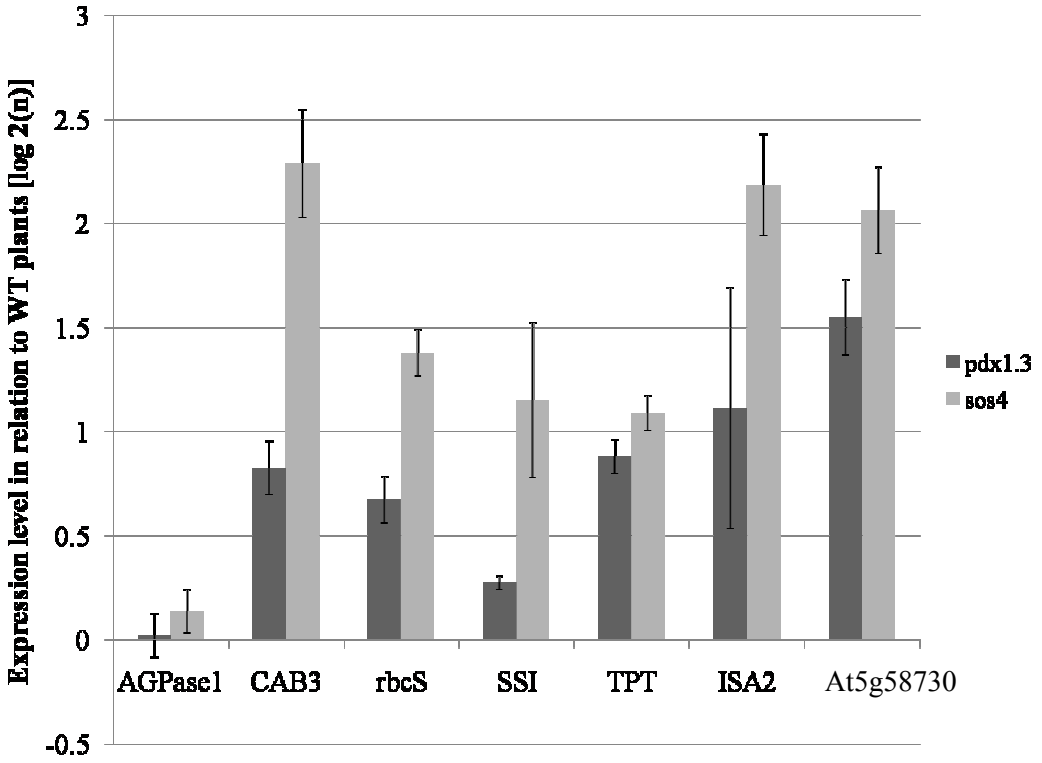


Figure 12. Transcript abundances of carbohydrate metabolic and photosynthetic genes in *pdx1.3* and *sos4* mutants relative to expression in wild type plants assayed by qRT-PCR. Genes assayed encode: ADP-glucose pyrophosphorylase (AGPase), chlorophyll AB-binding protein (CAB3), RuBisCO small subunit (rbcS), starch synthase I (SSI), triose phosphate transporter (TPT), isoamylase 2 (ISA2). Arabidopsis homologue (At5g58730) of the *E. coli PdxY* gene encoding a PL-specific kinase is also shown. Data represents log2(n) of genes in relation to wild type levels. Data represent one biological replicate with three technical replicates. Two biological replicates were performed with similar results. Error bars represent standard error of technical replicates.

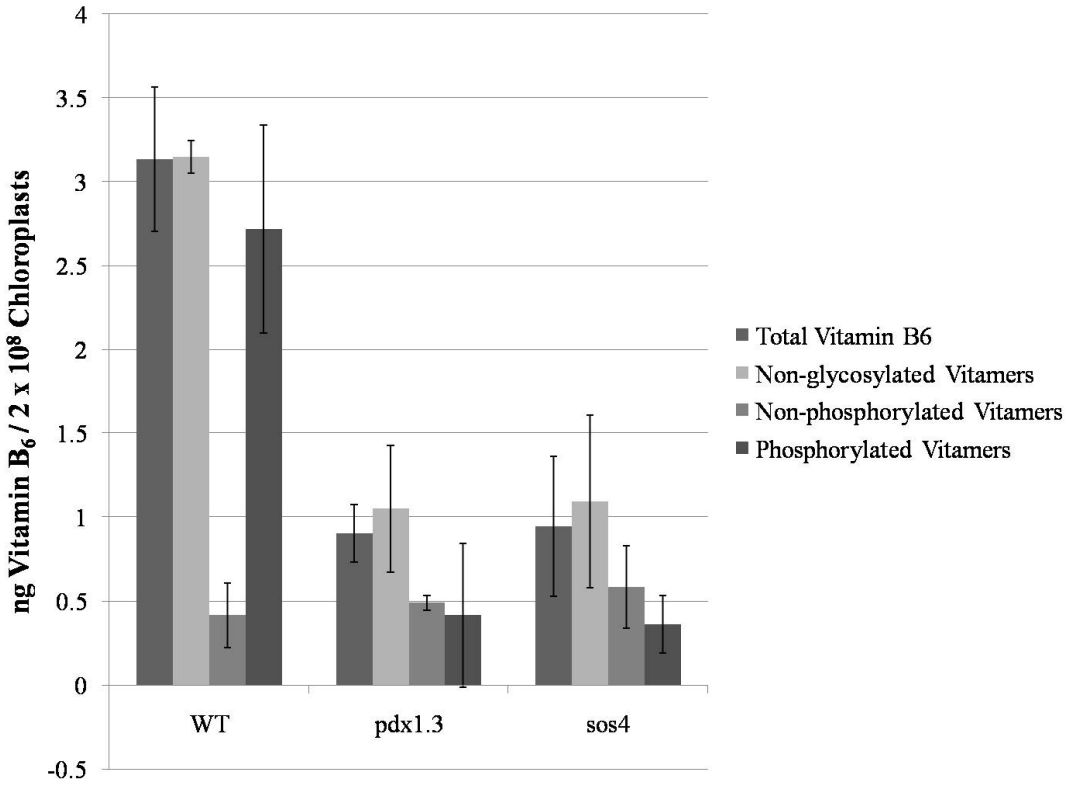


Figure 13. Vitamin B_6 content in chloroplasts of wild type, *pdx1.3* and *sos4* mutants. Chloroplasts were isolated from six week old plants after 20 hours of dark and vitamin B_6 was extracted as described in the methods. Total B_6 = extracts treated with acid phosphatase and β-glucosidase; free vitamers = extracts treated only with acid phosphatase; non-phosphorylated vitamers = extracts treated only with β-glucosidase; phosphorylated vitamers = difference between extracts treated with both enzymes and with β-glucosidase alone. Both *pdx1.3* and *sos4* mutants differ significantly from wild type in total B_6 levels due entirely to reduction in levels of phosphorylated vitamers. Data represent at least two independent biological replicates. Error bars represent standard errors. Means followed by the same letter are not statistically significant at $P \leq 0.05$ according to Duncan's multiple range test.

Table 1. Root growth of *pdx1.3* and *sos4* mutants on 100 mM sucrose, with and without vitamin B_6 supplementation. Seedlings were germinated on MS medium without supplementation and transferred to appropriate experimental conditions after one week of growth. Growth measurements were made from day of transfer.

	Root Length (mm) at 10 days ± SE		
B_6 supplement (2.5µM)	**Wild type**	***pdx1.3***	***sos4***
None	31.0 ± 1.7	7.2 ± 0.6	4.3 ± 0.3
Pyridoxine	25.2 ± 1.2	29.7 ± 3.0	3.4 ± 0.6
Pyridoxal	33.2 ± 2.0	28.2 ± 1.9	5.3 ± 1.2
Pyridoxal 5'-phosphate	30.5 ± 2.9	29.8 ± 1.7	4.2 ± 0.5

Table 2. Forward and reverse primers used for amplification of cDNA from Arabidopsis mutants for qRT-PCR.

Gene	Locus	Forward Primer	Reverse Primer
Actin1	AT2G37620	5'-TGGAACTGGAATGGTTAAGGCTGG-3'	5'-TCTCCAGAGTCGAGCACAATACCG-3'
AGPase	AT6G48300	5'-AGCCGCGTTATTTACCACCGTCTA-3'	5'-ATATGCAGGAACGGAGTCCAACCA-3'
CAB3	AT1G29910	5'-AGGTGAAGGAGCTCAAGAACGGAA-3'	5'-CCCATGCGTTGTTGTTGACTGGAT-3'
rbcS	AT5G38420	5'-TCCATCACAAGCAACGGAGGAAGA-3'	5'-TCCACTTGTTGCGGAGAAGGTAGT-3'
SSI	AT5G24300	5'-TTGTTCATGGCACTGGAGGACTCA-3'	5'-TTTCGACAAGGGAGTGAAGACCCA-3'
TPT	AT5G46610	5'-GGTCCGTGGGTCTTCCTAAAC-3'	5'-CCGCAGCGAAAGAGACATTGCTA-3'
ISA2	AT1G03310	5'-GTATTGGAGAGCTTGCGTTATTGG-3'	5'-GCGGATTTGTTTTAGCCTTCTTTG-3'
PDXY	AT5G58730	5'-TCACAACGAGTTCCATGAGCGTCT-3'	5'-ACAGGGACCGATCATCACAAGAGT-3'

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Chapter 4: Identification of protein-protein interactions of PDX1.3 through Yeast 2 Hybrid technology

INTRODUCTION

Because the *sos4* mutants of *Arabidopsis thaliana* accumulate increased levels of vitamin B_6 in whole leaf tissue and the *pdx1.3* mutants accumulated less vitamin B_6 than required for proper metabolism, it was unclear why both mutants shared so many phenotypes, many of which are consistent with a deficiency in vitamin B_6. In Chapter 3, evidence is presented to show that at least some of the shared phenotypes are due to a deficiency in phosphorylated vitamers in chloroplasts, but other phenotypes (most notably sucrose sensitivity of roots) cannot yet be explained. Another hypothesis that explains similar phenotypes shared between the two mutants is based on the idea that these mutants lack these proteins, and the proteins may play a role in metabolism outside their enzymatic function in the de novo and salvage pathways. Specifically, we hypothesized that PDX1.3 and SOS4 may either interact together or interact with other proteins and play a regulatory role in a common cellular process or protein complex.

Vitamin B_6 is involved in over one hundred different cellular reactions, including those of amino acid metabolism (Drewke and Leistner, 2001), fatty acid metabolism (Horrobin, 1993; Nakamura and Nara, 2004), carbohydrate metabolism

(Shimomura et al., 1980; Takagi et al., 1981; Withers et al., 1981; Fukui et al., 1982), heme and chlorophyll biosynthesis (Vavilin and Vermaas, 2002) and synthesis of ethylene (Tsang et al., 2003), an important plant hormone. In addition, vitamin B_6 has also been shown to be a potent antioxidant (Bilski et al., 2000; Chen and Xiong, 2005; Denslow et al., 2005; Seyithan, 2005) and a transcriptional regulator (Huq et al., 2007). Because it is a central metabolite involved in such a large number of cellular processes, we hypothesized that proteins of the metabolic pathways of vitamin B_6 may play other important roles within the cell, or serve as "moonlighting" proteins (Jeffery, 2003). Since shared phenotypes between the *pdx1.3* and *sos4* mutants include alterations in carbohydrate metabolism and decreased root growth when grown in the presence of increased levels of exogenous sucrose, we hypothesized, for example, that the PDX1.3 and SOS4 proteins may be involved in sucrose signaling and regulation of carbohydrate metabolism through protein-protein interactions.

This phenomenon of proteins playing multiple, unrelated roles within the cell is documented in several different organisms, including *Saccharomyces cerevisiae* and *A. thaliana.* In *S. cerevisiae,* Clf1p functions in both mRNA splicing processes and in the initiation of DNA replication (Ben-Yehuda et al., 2000; Russell et al., 2000; Jeffery, 2003). In *A. thaliana,* HXK1, hexokinase I, has been shown to play separable roles in glucose signaling and glucose metabolism (Moore et al., 2003;

Karve et al., 2008). Not only does hexokinase I function as a glucose sensor, but glucose itself has been shown to regulate genes, including those of carbohydrate metabolism, signal transduction and metabolite transport (Price et al., 2004). Mutants *pdx1.3* and *sos4* mutants also exhibit changes in transcriptional levels of carbohydrate metabolism genes. In addition, root growth of the *pdx1.3* and *sos4* mutants is severely reduced when grown in the presence of exogenous sucrose, indicating that vitamin B_6 or its metabolic proteins, PDX1.3 and/or SOS4, may play a role in sucrose sensing. The mechanism of the root sensitivity to sucrose in both of the mutants remains uncharacterized.

The common phenotypes associated with mutants of either PDX1.3 or SOS4 proteins might suggest that the regulatory function requires a direct protein:protein interaction with each other, or with other proteins in a common protein complex. PDX1.3 has been shown to interact with other PDX1 homologs and with PDX2 in the PLP synthase complex. In addition, PDX1.3 was identified in an interaction screen for proteins that interact with a scaffolding protein in the cell (Chen and Xiong, 2005). No other interacting partners of PDX1.3 are known in Arabidopsis. No protein interactions in *A. thaliana* have been reported for SOS4.

While not well characterized in Arabidopsis, a great deal is known about protein-protein interactions of proteins involved in pyridoxal metabolism in *S. cerevisiae*. These data are catalogued on an online database, www.biogrid.org. This

database suggested that PDX1.3 interacts with proteins outside of the vitamin B_6 de novo pathway, although no interactions were identified for SOS4. Therefore, a Yeast 2 Hybrid (Y2H) interaction screen was conducted with an Arabidopsis cDNA library to identify novel protein-protein interactions with the PDX1.3 protein.

METHODS

Y2H Assay

The yeast two-hybrid screen was conducted using the Clontech Matchmaker Library Construction and Screening Kit (Clontech; 630445), following the protocol provided by the manufacturer. PDX1.3 (At5g01410) cDNA was cloned into pGBKT7, a vector that contains the GAL4 DNA-binding domain and a gene for kanamycin resistance. A cDNA library was generated from RNA isolated from 6 week old adult leaf tissue using SMART (**S**witching **M**echanism **a**t 5' end of **R**NA **T**ranscript) technology (Zhu et al., 2001). This cDNA library was subsequently cloned into pGADT7, which carries the GAL4 activation domain and a gene for ampicillin resistance. The pGBKT7 vector containing PDX1.3 cDNA was transformed into yeast strain AH109 while the prey library was transformed into yeast strain Y187. The genotype of yeast strain AH109 is *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, LYS::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-*

GAL2$_{TATA}$*-ADE2, URA2::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ, MEL1*. The genotype of yeast strain Y187 is *MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, MEL1, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ.*

Transformed yeast were mated twice following manufacturer protocols, and putative positive interacting colonies were determine ermined by production of a blue color when grown in the presence of X-α-Gal (Figure 2). Plasmids from positive yeast colonies were isolated and transformed into Top 10 *E. coli* cells for plasmid propagation. Prey plasmids were selected for by growth on medium containing ampicillin. Plasmids from transformants were isolated by a miniprep procedure (Promega Wizard® *Plus* SV Minipreps DNA Purification System, Cat. # A1330), and the cDNA insert was sequenced to identify putative interacting partners.

Germination Assay

Wild type, *pdx1.3* and *sos4* mutants and segregating seed lines of T-DNA insertion lines SALK_068147, SALK_007079, and SALK_007080 were surface sterilized as described in Chapter 3. Seeds were suspended in sterile 0.1% agarose (top agar) and plated on Murashige and Skoog (MS) salts medium supplemented with 100 mM sucrose. No vitamins were added to the medium. Plates were stratified at 4˚C for 48 hours. Dark conditions were ensured by wrapping the plates in aluminum foil. Wrapped plates were moved to a 22˚C incubator for 48 hours, after which the

plates were unwrapped and incubated for one week under short day conditions (8 hours light/16 hours dark). Germination was assessed after one week of growth.

Root Growth Assay

Seedlings from wild type, *pdx1.3* and *sos4* mutants and segregating T-DNA insertion lines (SALK_068147, SALK_007079, and SALK_007080) from the germination assay were transferred to square plates containing MS medium supplemented with 100 mM sucrose and grown vertically under short day (8 hours) conditions. Root length was measured at 0 days, 4 days, and 8 days post transfer.

RESULTS

The yeast Biogrid database was mined for evidence of protein-protein interactions of homologues of proteins involved in vitamin B_6 metabolism. The database identifies two types of interaction, physical interactions and genetic interactions. Physical interactions are those interactions in which two proteins have been shown to physically interact with each other. Genetic interactions occur when a mutation in one gene influences (either enhances or reduces) a phenotype of an organism mutated in a second gene. A summary of the reactions identified is illustrated in Figure 1. Functions of the interacting proteins are summarized in Table

1. Homologs of PDX1 were shown to physically interact with a variety of proteins outside of the de novo pathway. By contrast, there was no evidence that BUD16, the SOS4 homolog, physically interacts with any proteins, thus PDX1.3 was used in the Y2H experiments.

Transformed yeast were mated twice following manufacturer protocols, and putative positive interacting colonies was determined by production of a blue color when grown in the presence of X-α-Gal (Figure 2). Two different proteins were pulled from the PDX1.3 protein interaction screen. One of these proteins is AT1G06680, a component of the PSII protein complex. Because this interaction was identified once in only one of the two matings performed, and because PDX1.3 has not been experimentally located within the chloroplast, it is unlikely that this protein interaction is a valid interaction. On the other hand, a second interacting protein, At3g28670, was identified in three separate instances from both matings and is therefore more likely a "true" interaction.

At3g28670 has been electronically annotated as a zinc binding oxidoreductase and has been found in plasma membrane fractions (Nuhse et al., 2007). Other than this cellular location and oxidoreductase annotation in the Gene Ontology database (http://www.geneontology.org/), this protein is largely uncharacterized. The gene has 12 exons and 11 introns, encoding a protein 491 amino acids in length with a predicted molecular weight of approximately 54 kDa. Although annotated as an

oxidoreductase, no conserved domains are identified using the Conserved Domain Database at the NCBI website (www.ncbi.nlm.nih.gov) (Marchler-Bauer et al., 2009). Online microarray data (www.genevestigator.com) indicates that this gene is expressed in all tissues, most highly in reproductive tissues, especially in pollen. In addition, this gene is expressed across all stages of development, particularly during flowering, seed set, and germination (Zimmermann et al., 2004; Hruz T, 2008).

Segregating seed stocks of T-DNA insertion mutants of At3g28670 were obtained and assayed to determine if they shared any common phenotypes with the *pdx1.3* mutant. Insertion mutants SALK_068147, SALK_007079, and SALK_007080 (Alonso et al., 2003) have insertions in exon regions of At3g28670. No phenotypes have been previously reported for any of the mutants.

Our results indicate that germination rates of segregating lines of these mutants did not differ from wild type plants, indicating that the mutation is not lethal (Table 2). In addition, preliminary assays of root growth of the germinated seedlings of segregating lines did not differ from wild type plants, and there was no evidence of root sensitivity to sucrose (Figure 3).

DISCUSSION

Analysis of data from the yeast Biogrid database identified several putative physical interactions between the PDX1 (SNZ) proteins and proteins outside of the

vitamin B_6 pathways. These include a number of proteins with putative regulatory roles including a kinase, GTP-binding protein, nuclear import protein, and a chaperonin among others (Table 1).

A promising novel protein-protein interaction of PDX1.3 was identified through the Y2H mating screen. PDX1.3 was found to interact with At3g28670 three times in two independent mating experiments. This protein shows no homology to any of the proteins identified as interacting with PDX1 (SNZ) in the Biogrid database. This putative interaction needs to be confirmed by reciprocal mating assays or by pull-down assay (Akamatsu et al., 2003; Leseberg et al., 2008).

Preliminary screens of segregating lines of three T-DNA insertion mutants in At3g28670 have not as yet shown any shared phenotypes with the *pdx1.3* mutants. Screening to obtain the homozygous mutant lines of all three polymorphisms is in progress. Further characterization of the mutant phenotypes is required before making final conclusions regarding phenotypes of insertion mutants of At3g28670.

Although further work must be conducted to verify the protein interaction of At3g28670 with PDX1.3, the identification of the putative protein interaction is evidence that the PDX1 proteins may be interacting with proteins outside of the PLP synthase complex. The PLP synthase protein complex has been shown to be localized to both the cytoplasm and cellular membranes (Chen and Xiong, 2005; Titiz et al., 2006; Denslow et al., 2007). The PLP synthase complex itself is not a

membrane protein, and association with a membrane protein like At3g28670 might explain localization to membrane structures within the cell.

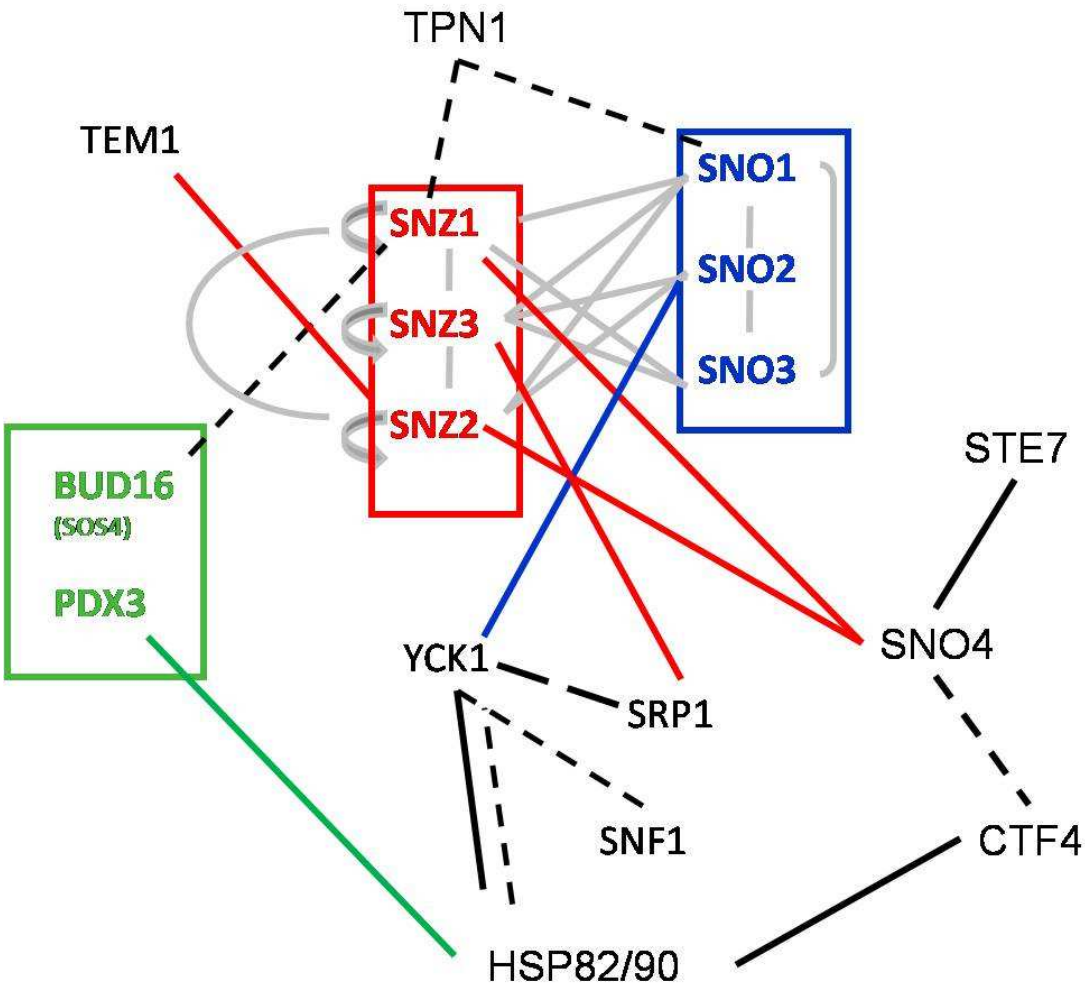


Figure 1. Protein-protein interactions of vitamin B_6 metabolic pathway genes in *S. cerevisiae* reported by the Biogrid database. SNZ proteins are homologous to PDX1 proteins and are represented in red. SNO proteins are homologous to the PDX2 protein and are represented in blue. BUD16 (SOS4 homolog) and PDX3 (PDX3 homolog) proteins are proteins in the salvage pathway and are represented in green. Solid lines indicate physical interactions, while dashed lines indicate genetic interactions. Arrows indicate interaction with self. See Table 1 for a list of interacting proteins, functions and interaction types. All data was obtained from the Biogrid database (www.BioGrid.org). Interactions shown above are not necessarily the only interactions experimentally confirmed for each protein.

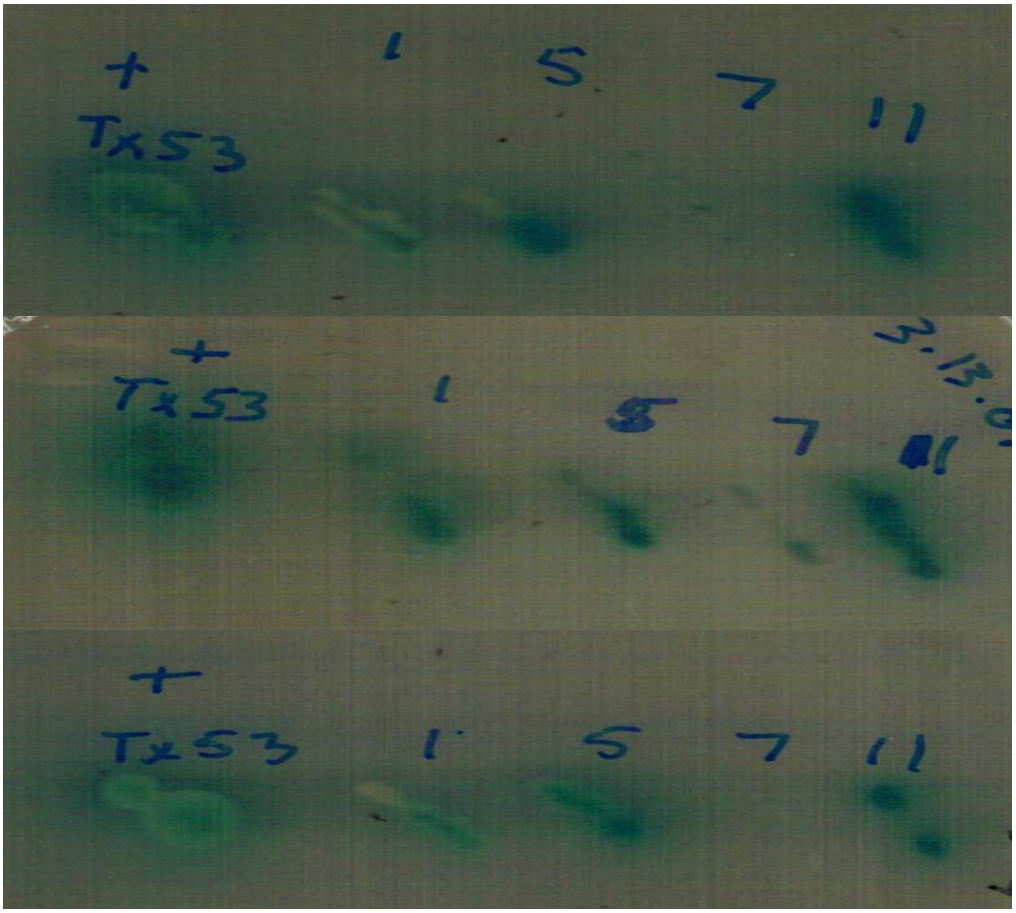
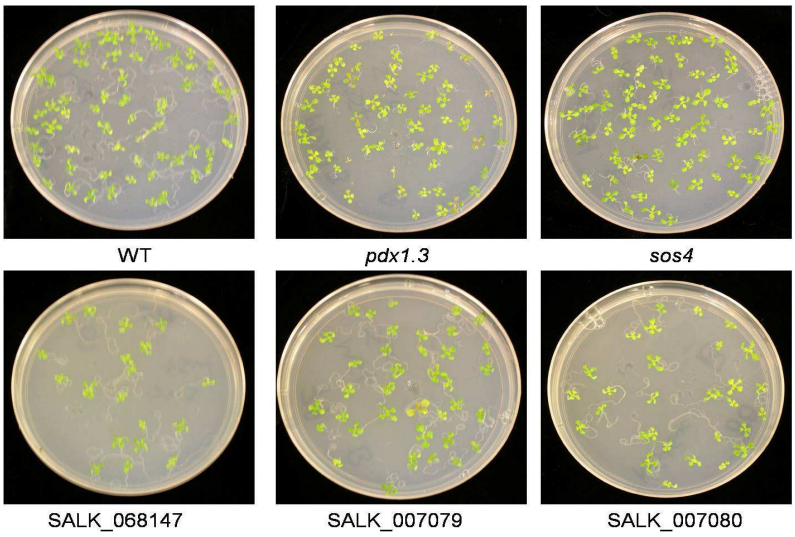


Figure 2. Positive interactions in Yeast Two Hybrid Screen. Bait vector contained cDNA of At5g01410. cDNA in the prey vector was sequenced and correspond to the following gene loci: Colony 1 - At1g06680, Colony 5 – At3g28670, Colony 7 – At3g28670, Colony 11 – At3g28670. Tx53 represents the positive protein interaction control. Colonies 1 and 5 were isolated during the first mating experiment, while colonies seven and eleven were isolated in a subsequent mating.

Figure 3. Germination and root growth of At3g28670 SALK T-DNA insertion mutants. A. Wild type, *pdx1.3* and *sos4* mutants and SALK T-DNA insertion mutants of At3g28670 germinated on MS medium supplemented with 100 mM sucrose. Germination rates are shown in Table 2. Note the short roots of the *pdx1.3* and *sos4* mutants. The SALK T-DNA insertion mutants of AT3g28670 do not exhibit the short root phenotype of the *pdx1.3* and *sos4* mutant lines. **B.** Root growth of wild type, *pdx1.3* and *sos4* mutants and SALK T-DNA insertion mutants of At3g28670 grown on MS medium supplemented with 100 mM sucrose. Root growth of SALK T-DNA insertion mutants was comparable to wild type plants. This data represents one experiment with six seedlings of each plant line. Error bars represent standard error.

A.


WT
pdx1.3
sos4
SALK_068147
SALK_007079
SALK_007080

B.

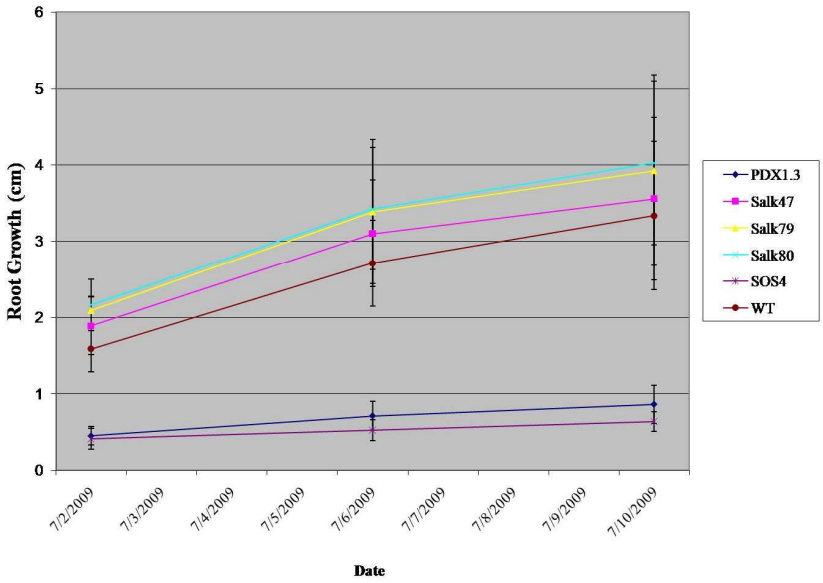

Root Growth (cm)
0
1
2
3
4
5
6
7/2/2009
7/3/2009
7/4/2009
7/5/2009
7/6/2009
7/7/2009
7/8/2009
7/9/2009
7/10/2009
Date
PDX1.3
Salk47
Salk79
Salk80
SOS4
WT

Table 1: Identification and functions of proteins in the yeast protein interaction model (Figure 1). Physical interactions listed in the table are in bold; others are genetic interactions.

Protein	Function	Associating protein	Interaction Type[a]
CTF4	Associated with chromatin; functions in sister chromatid cohesion	**HSP82/90**	**Affinity Capture – MS**
		SNO4	Synthetic Growth Defect
HSP82/90	Chaperonin involved in pheromone signaling; facilitates telomerase binding	**YCK1**	**Yeast 2 Hybrid** Synthetic Growth Defect
		PDX3	**Yeast 2 Hybrid**
		SNF1	Synthetic Lethality
		CTF4	**Affinity Capture - MS**
SNO4	Possible chaperonin; may play a role in pyridoxine metabolism	**SNZ2**	**Yeast 2 Hybrid**
		SNZ1	**Yeast 2 Hybrid**
		SNZ3	**Yeast 2 Hybrid**
		STE7	**PCA**
		CTF4	Synthetic growth defect
SRP1	Mediates import of nuclear proteins by binding the nuclear localization signal	**YCK1**	**Biochemical Activity**
		SNZ3	**Yeast 2 Hybrid**
STE7	MAPKK involved in pheromone signaling and the pseudohyphal/invasive growth pathway	**SNO4**	**PCA**
TEM1	GTP binding protein; involved in termination of M-phase	**SNZ2**	**Yeast 2 Hybrid**
TPN1	Vitamin B_6 transport protein	**SNZ1**	Synthetic Lethality
		SNO1	Synthetic Lethality
YCK1	Plasma membrane casein kinase I isoform; involved in septin assembly and endocytosis	**SRP1**	**Biochemical Activity**
		SNO2	**Affinity Capture - MS**
		HSP82/90	**Yeast 2 Hybrid** Synthetic Growth defect
		SNF1	Dosage Rescue

Table 1 (Continued)

Protein	Function	Associating protein	Interaction Type[a]
SNO1	PDX2 homolog; component of the PLP synthase complex	**SNO2**	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNO3	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNZ1	**Yeast 2 Hybrid** **Reconstituted Complex** Synthetic Rescue Phenotype Enhancement
		SNZ2	**Yeast 2 Hybrid**
		SNZ3	**Yeast 2 Hybrid**
		TPN1	Synthetic Lethality
SNO2	PDX2 homolog; component of the PLP synthase complex	**SNO1**	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNO3	Phenotypic Enhancement
		SNZ3	**Yeast 2 Hybrid**
		SNZ2	**Yeast 2 Hybrid**
		YCK1	**Affinity Capture - MS**
SNO3	PDX2 homolog; component of the PLP synthase complex	**SNZ1**	**Yeast 2 Hybrid**
		SNZ3	**Yeast 2 Hybrid**
		SNO2	Phenotypic Enhancement
		SNO1	**Yeast 2 Hybrid** Phenotypic Enhancement
SNZ1	PDX1 homolog; component of the PLP synthase complex	**SNZ1**	**Yeast 2 Hybrid** **PCA**
		SNZ3	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNZ2	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNO1	**Yeast 2 Hybrid** **Reconstituted Complex** Synthetic Rescue Phenotypic Enhancement
		SNO2	**Yeast 2 Hybrid**
		SNO3	**Yeast 2 Hybrid**
		SNO4	**Yeast 2 Hybrid**
		BUD16	Synthetic Lethality
		TPN1	Synthetic Lethality

Table 1 (Continued)

Protein	Function	Associating protein	Interaction Typea
SNZ2	PDX2 homolog; component of the PLP synthase complex	**SNZ2**	**Protein-Peptide** **Yeast 2 Hybrid**
		SNZ3	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNZ1	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNO1	**Yeast 2 Hybrid**
		SNO2	**Yeast 2 Hybrid**
		SNO4	**Yeast 2 Hybrid**
		TEM1	**Yeast 2 Hybrid**
SNZ3	PDX2 homolog; component of the PLP synthase complex	**SNZ3**	**Protein-Peptide**
		SNZ1	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNZ2	**Yeast 2 Hybrid** Phenotypic Enhancement
		SNO1	**Yeast 2 Hybrid**
		SNO2	**Yeast 2 Hybrid**
		SNO3	**Yeast 2 Hybrid**
		SRP1	**Yeast 2 Hybrid**
		SNO4	**Yeast 2 Hybrid**
BUD16	SOS4 homolog; involved in bud site selection and in genome integrity; phosphorylates PLP	**SNZ1**	Synthetic Lethality
PDX3	Pyridoxine (pyridoxamine) oxidase;	**HSP82/90**	**Yeast 2 Hybrid**

[a] Definitions of interactions:

Physical Interactions:

Yeast 2 Hybrid – bait and prey proteins cloned into separate vectors and interaction measured by reporter gene activation
Reconstituted Complex – detection of interactions of two proteins in vitro

PCA – similar to Yeast 2 Hybrid assay, bait and prey proteins cloned into separate vectors, interaction results in activity of reporter molecule
Protein Peptide – detection of interaction between a protein and a peptide from an interaction partner (such as in a bioengineered phage molecule)
Affinity Capture – MS – bait protein is affinity captured and the associated protein is identified through mass spectrometry
Biochemical interaction – physical interaction is inferred due to biochemical effect of one protein on another.

Genetic Interactions:

Synthetic Rescue – genetic interaction inferred when mutations or deletions in one gene rescues lethality or growth defect of a different strain with a mutation or defect in another gene
Phenotypic enhancement – genetic interaction inferred when mutation or overexpression of one gene enhances the phenotype associated with overexpression or mutation of another gene in a different strain
Synthetic lethality – genetic interaction is inferred when mutations in separate genes cause minimal phenotypic changes by themselves, but when combined in the same organism causes lethality
Synthetic Growth Defect – similar to synthetic lethality, but combination of mutations of separate genes results in significant growth defect rather than lethality
Dosage Rescue – genetic interaction is inferred when overexpression or increased dosage of one gene rescues growth defect or lethality in a strain with a mutation in a different gene.

Data and interactions obtained from www.biogrid.org.

Table 2. Germination rates of wild type, *pdx1.3* and *sos4* mutant seeds and seeds of SALK T-DNA insertion mutants of At3g28670

Seed Line	Plants Germinated	Seeds Plated	Germination Rate
Wild type	83	91	91.2%
pdx1.3	65	79	82.3%
sos4	73	77	94.8%
SALK_068147	28	32	87.5%
SALK_007079	46	47	97.9%
SALK_007080	37	40	92.5%

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