Vitamer Levels, Stress Response, Enzyme Activity, and Gene Regulation of Arabidopsis Lines Mutant in the Pyridoxine/Pyridoxamine 5′-Phosphate Oxidase (*PDX3*) and the Pyridoxal Kinase (*SOS4*) Genes Involved in the Vitamin B_6 Salvage Pathway[1][W][OA]

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PDX3 and *SALT OVERLY SENSITIVE4* (*SOS4*), encoding pyridoxine/pyridoxamine 5′-phosphate oxidase and pyridoxal kinase, respectively, are the only known genes involved in the salvage pathway of pyridoxal 5′-phosphate in plants. In this study, we determined the phenotype, stress responses, vitamer levels, and regulation of the vitamin B_6 pathway genes in Arabidopsis (*Arabidopsis thaliana*) plants mutant in *PDX3* and *SOS4*. *sos4* mutant plants showed a distinct phenotype characterized by chlorosis and reduced plant size, as well as hypersensitivity to sucrose in addition to the previously noted NaCl sensitivity. This mutant had higher levels of pyridoxine, pyridoxamine, and pyridoxal 5′-phosphate than the wild type, reflected in an increase in total vitamin B_6 observed through HPLC analysis and yeast bioassay. The *sos4* mutant showed increased activity of PDX3 as well as of the B_6 de novo pathway enzyme PDX1, correlating with increased total B_6 levels. Two independent lines with T-DNA insertions in the promoter region of PDX3 (*pdx3-1* and *pdx3-2*) had decreased PDX3 activity. Both also had decreased activity of PDX1, which correlated with lower levels of total vitamin B_6 observed using the yeast bioassay; however, no differences were noted in levels of individual vitamers by HPLC analysis. Both *pdx3* mutants showed growth reduction in vitro and in vivo as well as an inability to increase growth under high light conditions. Increased expression of salvage and some of the de novo pathway genes was observed in both the *pdx3* and *sos4* mutants. In all mutants, increased expression was more dramatic for the salvage pathway genes.

Pyridoxine/pyridoxamine 5′-P (PNP/PMP) oxidase and pyridoxal (PL) kinase, encoded in Arabidopsis (*Arabidopsis thaliana*) by the *PDX3* (Sang et al., 2007) and the *SALT OVERLY SENSITIVE4* (*SOS4*; Shi and Zhu, 2002) genes, respectively, are key enzymes involved in the biosynthetic pathway leading to pyridoxal 5′-P (PLP). PLP is the active coenzyme product of the vitamin B_6 pathway and is essential in many biochemical reactions, including decarboxylation, transamination, deamination, racemization, and trans-sulfuration reactions associated primarily with amino acid synthesis (Drewke and Leistner, 2001). PLP is also involved in enzymes that catalyze some steps in carbohydrate and lipid metabolism and is a cofactor for aminocyclopropane-1-carboxylate synthase. Moreover, vitamin B_6 has been linked to resistance to oxidative and environmental stresses and is an efficient singlet oxygen quencher and antioxidant (Shi et al., 2002; Denslow et al., 2005, 2007). It is required for postembryonic root development and root hair development in plants (Shi and Zhu, 2002; Chen and Xiong, 2005; Denslow et al., 2005, 2007; Titiz et al., 2006).

Organisms can synthesis PLP de novo or by a salvage pathway. *Escherichia coli* and other proteobacteria synthesize PLP de novo by the well-studied *pdxA*/*pdxJ* pathway (Roa et al., 1989; Hill et al., 1996; Man et al., 1996; Laber et al., 1999). In this pathway, PNP, a direct biosynthetic intermediate leading to PLP, is synthesized by the condensation of 4-(phosphohydroxy)-L-Thr and 1-deoxy-D-xylulose-5-P, catalyzed by the enzymes PdxA and PdxJ. PNP is then oxidized by a PNP/PMP oxidase encoded by *pdxH* to form PLP (Laber et al., 1999). An alternative de novo PLP biosynthesis pathway that involves two different proteins, PDX1 and PDX2, has also been characterized and is present in the majority of organisms that can produce vitamin B_6 de novo, including plants, fungi, archeabacteria, and most bacteria (Ehrenshaft et al., 1999; Osmani et al., 1999; Ehrenshaft and Daub, 2001; Mittenhuber, 2001). In this pathway, proteins encoded by the genes *PDX1* and *PDX2* form a

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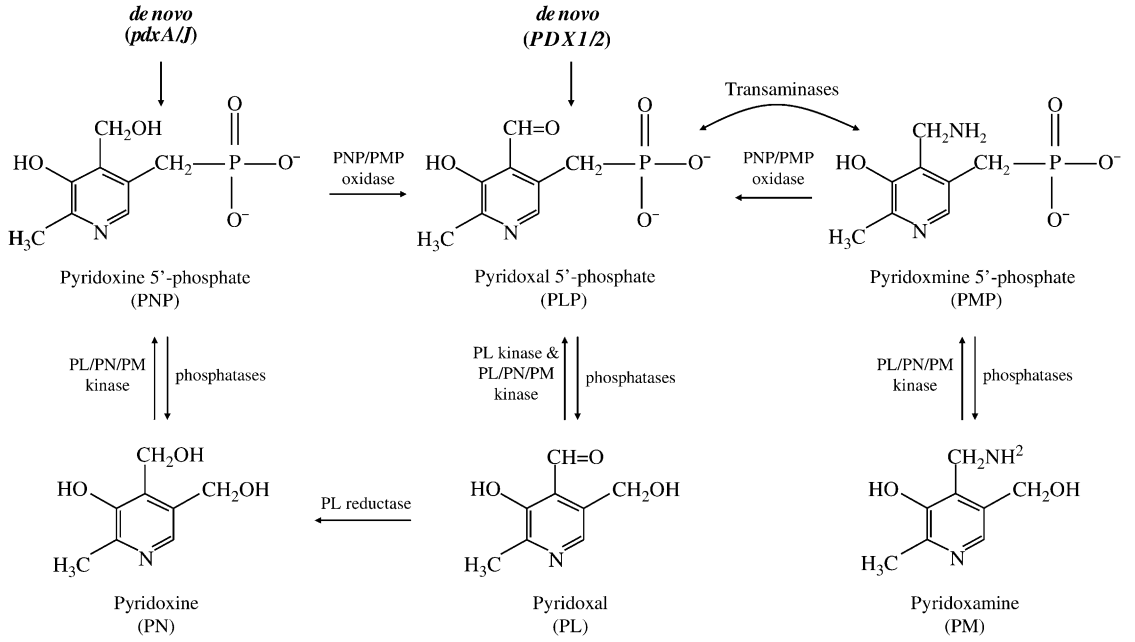

Figure 1. Vitamin B_6 salvage pathway. PLP is produced both via the de novo pathway in organisms containing *PDX1* and *PDX2* as well as by conversion from PNP or PMP via the PNP/PMP oxidase. PMP oxidation can also be catalyzed by transaminases. PN, PM, and PL are phosphorylated by the action of a PL/PN/PM kinase or by an additional PL-specific kinase for PLP. Phosphorylated derivatives are dephosphorylated by phosphatases. PL can also be converted to PN by a PL reductase. This pathway is based on data from *E. coli*, yeast, *B. subtilis*, and mammals.

complex that has Gln amidotransferase activity (Belitsky, 2004). This enzyme complex catalyzes a reaction where Gln plus either Rib 5-P or ribulose 5-P and either dihydroxyacetone phosphate or glyceraldehyde 3-P are used to produce PLP, the first B_6 vitamer synthesized in this pathway (Burns et al., 2005; Raschle et al., 2005). *PDX1* and *PDX2* were recently isolated and characterized in tobacco (*Nicotiana tabacum*; Denslow et al., 2005) and Arabidopsis (Chen and Xiong, 2005; Tambasco-Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Denslow et al., 2007). Two *PDX1* sequences were recovered from tobacco, suggesting either two copies or two alleles; one copy of *PDX2* was identified. In Arabidopsis, there is a single copy of *PDX2* on chromosome 5 and three homologs of *PDX1* located on chromosomes 2, 3, and 5, referred to as *PDX1.1*, *PDX1.2*, and *PDX1.3*, respectively.

Many organisms, including those that cannot synthesize PNP or PLP de novo, contain a salvage pathway that interconverts between the different vitamers (Fig. 1). In this pathway, the nonphosphorylated forms of vitamin B_6 are converted to PLP by the action of kinases that phosphorylate pyridoxine (PN), pyridoxamine (PM), and PL to form PNP, PMP, and PLP, respectively, and an oxidase that catalyzes the oxidation of PNP and PMP to form PLP. Alternatively, PMP oxidation can also be catalyzed by transaminases (Mittenhuber, 2001). The phosphorylated forms can be hydrolyzed by phosphatases to restore the free vitamers and maintain PLP homoeostasis (McCormick and Chen, 1999; Mittenhuber, 2001). Additionally, a PL reductase that reduces PL to PN has been suggested to be involved in the vitamin B_6 salvage pathway (Guirard and Snell, 1988; Nakano et al., 1999; Morita et al., 2004).

The vitamin B_6 salvage pathway enzymes and genes have been characterized in several organisms. In *E. coli*, two kinases with different substrate specificity

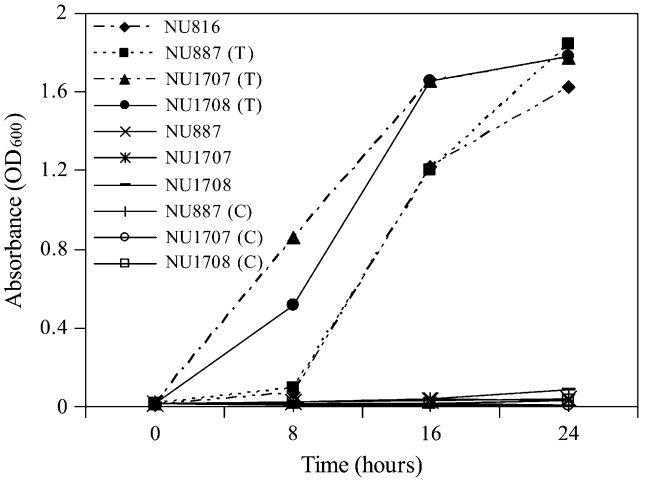

Figure 2. Growth of *E. coli pdxH* mutants, mutants transformed with Arabidopsis *PDX3*, and the parental strain on minimal medium. NU887, NU1706, and NU1708, *pdxH E. coli* mutants; (T), *pdxH E. coli* mutants transformed with the Arabidopsis *PDX3* gene; NU816, parental strain; (C), *pdxH E. coli* mutants transformed with vector control. Mutants were grown on minimal medium (liquid EM plus 0.01 mM $FeSO_4$).

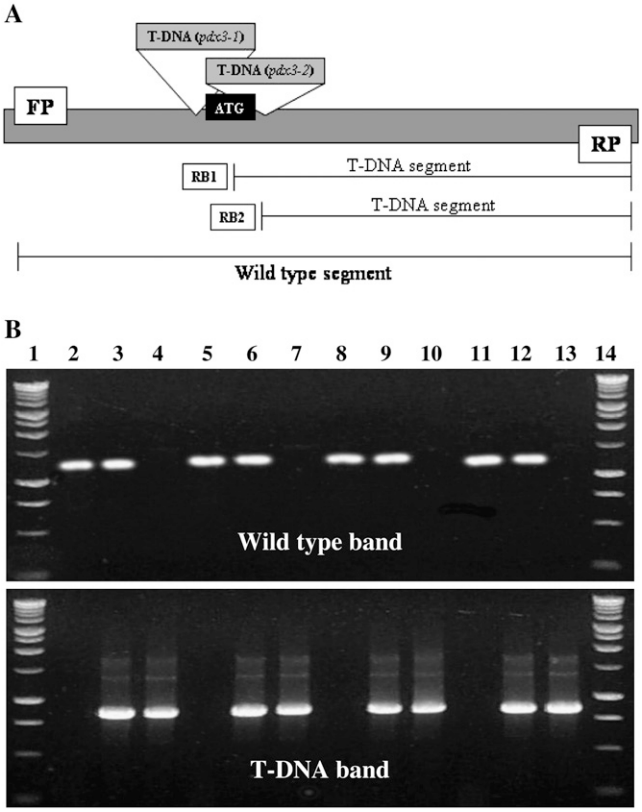

Figure 3. A, Diagram of the location of the T-DNA insertions in the *pdx3* mutant lines (*pdx3-1* and *pdx3-2*) within the *PDX3* gene. FP and RP indicate forward and reverse primers used to amplify the 1,200-bp wild-type *PDX3* segment. RB1 and RB2 are the T-DNA right-border primers used with RP to amplify the approximately 800-bp segments for the T-DNA insertion mutants. B, Screening of T-DNA *pdx3* insertion lines by PCR. DNA from plants segregating for the T-DNA insertion was amplified with the FP and RP primers (top) to amplify the 1,200-bp wild-type sequence or with the RB1 or RB2 primers plus the RP primer (bottom) to amplify the approximately 800-bp sequence resulting from the T-DNA insertions. Plants lacking T-DNA insertions are identified by the amplification of only the 1,200-bp band; homozygous T-DNA mutants have only the 800-bp band and heterozygous plants have both bands. Lanes 1 and 14, 1-kb ladder; lanes 2, 5, 8, and 11, plants lacking the T-DNA insertion (wild type); lanes 3 and 6, *pdx3-1* heterozygous plants; lanes 9 and 12, *pdx3-2* heterozygous plants; lanes 4 and 7, *pdx3-1* homozygous mutants; lines 10 and 13, *pdx3-2* homozygous mutants. PCR products were visualized on 1% agarose gels. The difference in distance between the T-DNA insertion in the *pdx3-1* and *pdx3-2* mutant lines is only 38 bp, resulting in bands that are not distinguishable on the gel.

have been identified. PN/PM/PL kinase (PdxK) phosphorylates PN, PM, and PL to form PNP, PMP, and PLP, respectively, whereas PL kinase (PdxY) specifically phosphorylates PL (Yang et al., 1996, 1998). The oxidation of PNP and PMP to form PLP is carried out by PdxH, the same oxidase involved in the de novo pathway (Lam and Winkler, 1992). Kinases and oxidases with sequence and functional homology to PdxK and PdxH have also been identified and thoroughly characterized in mammals (Kazarinoff and McCormick, 1975; Kwok and Churchich, 1980; Choi et al., 1987; Hanna et al., 1997). However, little is known about the vitamin B_6 genes involved in the salvage pathway in organisms that synthesize PLP de novo by the PDX1/PDX2 pathway. A kinase (ThiD) involved in thiamine biosynthesis in *Bacillus subtilis* was recently characterized and shown also to have PN, PM, and PL kinase activity (Park et al., 2004). A PL kinase has also been identified and characterized in plants (Lum et al., 2002; Shi et al., 2002; Wang et al., 2004). This enzyme, identified in Arabidopsis by its ability to complement *E. coli pdxK* mutants (Shi et al., 2002), is encoded by the *SOS4* gene associated with NaCl tolerance and root hair development in Arabidopsis (Shi et al., 2002; Shi and Zhu, 2002) and wheat (*Triticum aestivum*; Wang et al., 2004). A PNP/PMP oxidase, PDX3, has been also identified and characterized in yeast (*Saccharomyces cerevisiae*; Loubbardi et al., 1995), and recently in Arabidopsis by complementation of a yeast *pdx3* mutant (Sang et al., 2007).

In this study, we confirm, by complementation of *pdxH E. coli* mutants, the recently published identity (Sang et al., 2007) of the Arabidopsis locus At5g49970 as the gene encoding PDX3, the PNP/PMP oxidase. In addition, we report the effects on phenotype, vitamer levels, stress responses, enzyme activity, and regulation of salvage and de novo pathway genes, resulting

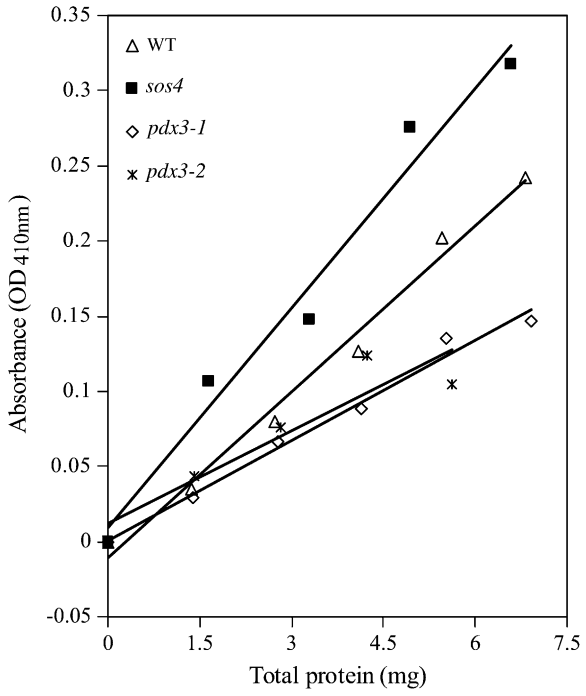

Figure 4. PDX3 activity in Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT). The activity of PDX3 was measured in crude extracts of the mutant lines and WT by determining the amount of PLP formed with a colorimetric assay after a 1-h incubation of enzyme reactions containing increasing amounts of total protein. PDX3 activity data were analyzed with a regression line adjusted to the absorbance values obtained for the increasing amounts of total protein.

Table I. *Phenotypic characterization of Arabidopsis pdx3 and sos4 mutants compared to wild-type Arabidopsis ecotype Col-0 (WT)*

	First Flower Buds Visible (Days)[a,b]	First Flower Open (Days)[a,b]	No. of Seeds/Silique[a,b]	Percentage of Seed Germination
WT	30.3 b	45.2 a	34.5 a	95.8%
sos4	31.8 a	47.0 a	31.2 bc	96.5%
pdx3-1	30.3 b	43.9 a	30.4 c	95.1%
pdx3-2	30.3 b	45.6 a	34.0 ab	95.5%

[a]Plants were monitored from seed germination to senescence. [b]Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

from mutations in *pdx3* and *sos4*. In addition to the previously reported sensitivity of the *sos4* mutant to salt, we show that this mutant has root-specific sensitivity to Suc. Analysis of vitamer levels in the *sos4* mutant identified an unexpected increase in PLP levels along with predicted increases in PM and PN. The elevated B_6 levels correlated with increased enzyme activity of both PDX1 and PDX3, as well as increased expression of the encoding genes. By contrast, *pdx3* mutant lines have decreased total vitamin B_6 levels, which correlate with decreased PDX1 enzyme activity but not with the increased *PDX1* and *SOS4* gene expression seen in the *pdx3* mutants. Gene expression cannot explain the differential effect on B_6 vitamer levels between the mutants.

RESULTS

Arabidopsis *PDX3* Gene Complements *pdxH*

Identity of At5g49970 as encoding a PNP/PMP oxidase was determined by complementation of *E. coli pdxH* mutants. As shown in Figure 2, wild-type growth was restored in all *pdxH E. coli* mutants (NU887, NU1707, and NU1708). Only the mutants transformed with *PDX3* were capable of growing on minimal medium without PL and had comparable growth to the parental strain NU816. These results confirm the report of Sang et al. (2007) and also show that *PDX3* from Arabidopsis is functional in *E. coli*.

Recovery of *pdx3* Homozygous T-DNA Insertion Mutant Lines

Plants from Arabidopsis ecotype Columbia (Col-0) with T-DNA insertions located in two different sites of the *PDX3* promoter region (SALK_060749 and SALK_149382, named *pdx3-1* and *pdx3-2*, respectively; Fig. 3A) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous mutant plants were differentiated from wild-type and heterozygous plants by the presence of a single band of approximately 800 bp (Fig. 3B), compared to the presence of only a single 1,200-bp band in wild-type plants and the presence of both bands in heterozygous plants. The difference in distance between the T-DNA insertion in the *pdx3-1* and *pdx3-2* mutant lines is only 38 bp; therefore, the bands amplified in the *pdx3-1* and *pdx3-2* homozygous lines are indistinguishable in the gel (Fig. 3B).

PDX3 Gene Expression and Enzyme Activity in Insertion Mutants

Expression of *PDX3* in the *pdx3-1* and *pdx3-2* mutant lines was assayed by quantitative real-time PCR (qRT-PCR). No decrease was seen in *PDX3* expression in the *pdx3-1* mutant, but the *pdx3-2* mutant showed a 70% decrease in *PDX3* expression. PDX3 enzyme activity was then assayed in both mutant lines. We were unable to detect activity in the wild-type strain using the assay developed by Sang et al. (2007) for the purified Arabidopsis enzyme; however, we were able to measure activity using methods developed for PNP/PMP oxidase enzymes from *E. coli* and rabbit liver and brain tissues (Wada and Snell, 1961; Zhao

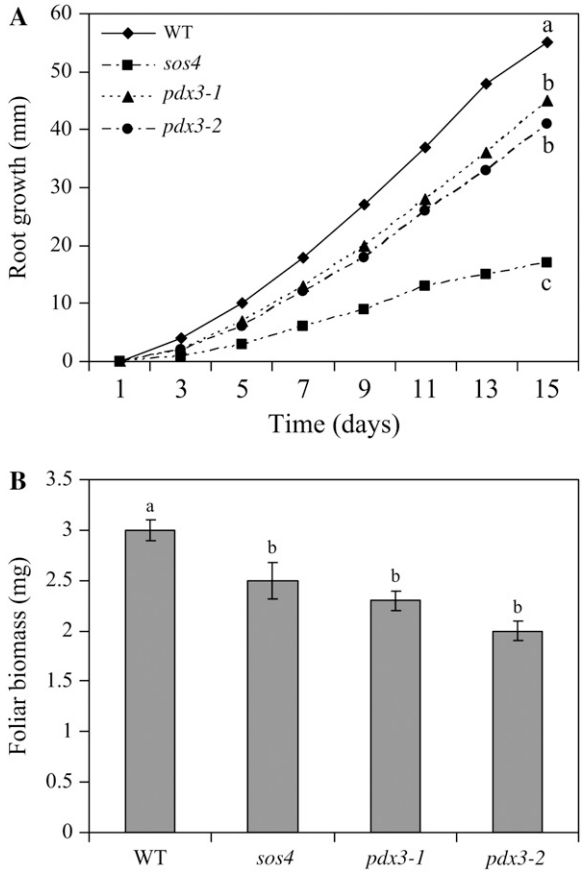

Figure 5. In vitro root growth and foliar biomass of Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT). A and B, Root growth (A) and foliar biomass (B) of plants grown on Murashige and Skoog medium. Root length was measured every other day for 15 d. Biomass (wet weight) was determined at 15 d. Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

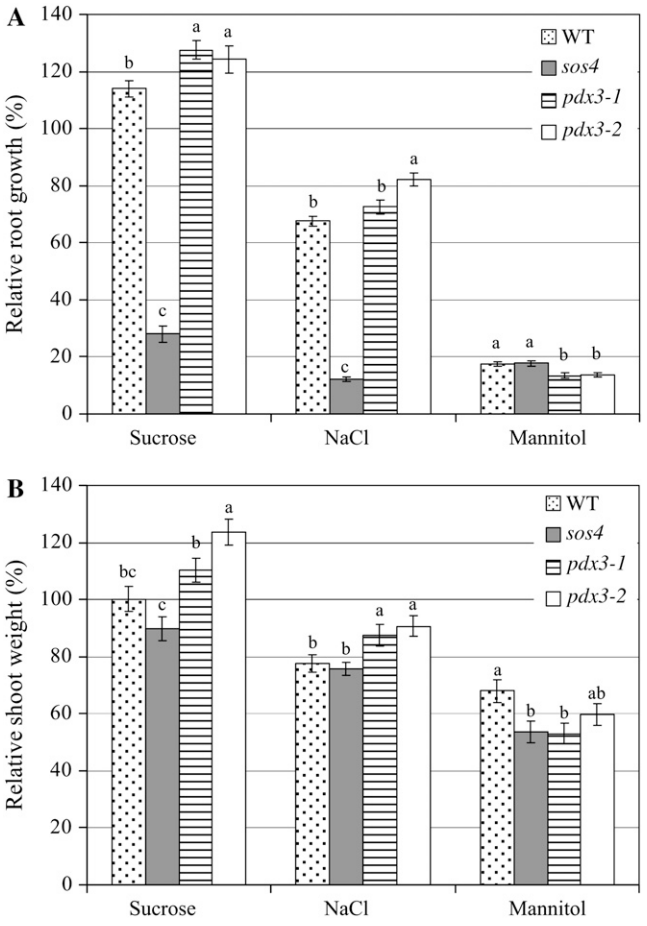

Figure 6. Sensitivity of Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT) to Suc, NaCl, and mannitol. A and B, Relative root growth (A) and relative shoot weight (B) of plants grown on Murashige and Skoog medium amended with 100 mM of Suc, NaCl, or mannitol; values shown are relative to the growth of plants of the same line on Murashige and Skoog medium alone. Roots were measured every other day for 15 d. Shoot weight was determined at 15 d. Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

and Winkler, 1995). Results are shown in Figure 4. Both *pdx3* mutants had lower oxidase activity than the wild type, with approximately one-half the activity at the highest amount of protein assayed. In spite of the different expression measured by qRT-PCR, oxidase activities in the two insertion lines were indistinguishable from each other. As a comparison, PDX3 activity was also assayed in the *sos4* mutant. This mutant showed higher oxidase activity than the wild type or either of the *pdx3* mutants.

Phenotypic Analysis of Mutant Lines

Phenotypes of the *pdx3* and *sos4* mutant lines were assessed both in soil and in vitro. In soil, *pdx3* mutant lines did not show a distinct phenotype in contrast to *sos4* mutant plants, which were chlorotic and had reduced plant size. The first flower buds were visible in *sos4* mutant plants before those in wild-type and *pdx3* mutant plants; however, there were no significant differences in the number of days to the first open flower between any of the mutant lines and the wild type (Table I). Only *pdx3-1* mutant plants, and not those of *pdx3-2*, showed significant reduction in the number of seeds per silique as compared to the wild type.

Seed germination and early seedling growth were assessed in vitro. None of the mutant lines showed differences in percent seed germination (Table I). Seedlings of both *pdx3* and *sos4* mutants had reduced root growth and foliar biomass compared to the wild type (Fig. 5). At day 15, *pdx3-1* and *pdx3-2* mutant lines showed, respectively, statistically significant 19% and 25% reduction in root growth and 23% and 33% reduction in foliar biomass. The *sos4* mutant plants showed the greatest reduction in root growth of over 69% at day 15. Foliar biomass in the *sos4* mutants was also significantly reduced (17%).

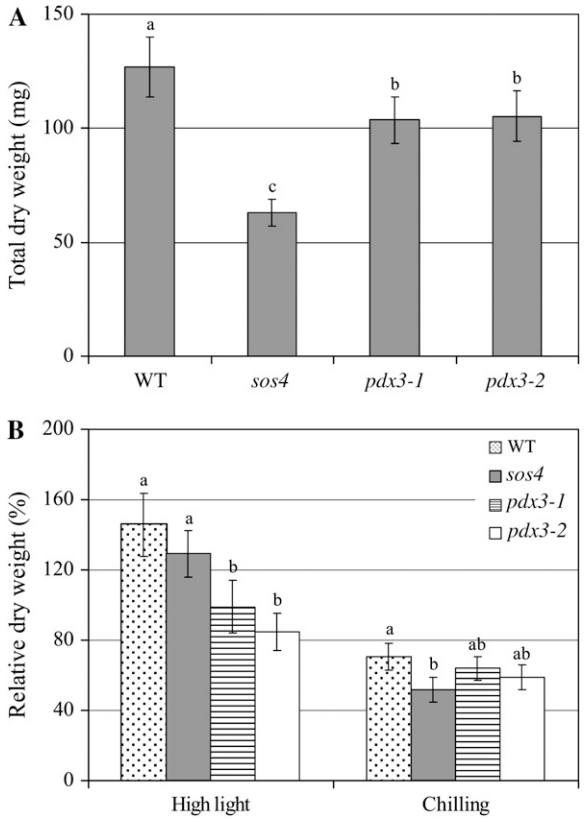

Figure 7. Response of Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT) to high light and chilling conditions. A, Total dry weight of mutant and WT plants grown under control conditions (20°C, 8-h photoperiod, 200 μmol s^{-1} m^{-2} light) for 3 weeks. B, Relative dry weight of mutant and WT plants exposed to high light (20°C, 8-h photoperiod, 1,000 μmol s^{-1} m^{-2}) and chilling (5°C, 8-h photoperiod, 200 μmol s^{-1} m^{-2} light) conditions. Dry weight is relative to the dry weight of plants of the same line grown under control conditions. Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

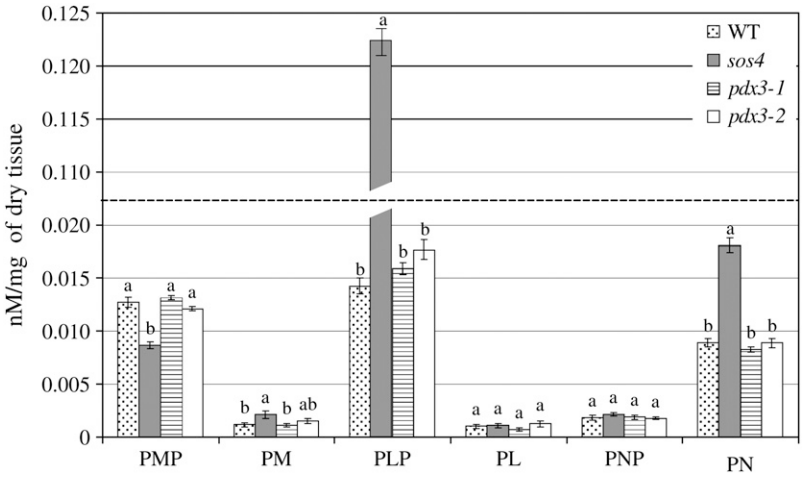

Figure 8. Levels of vitamin B_6 vitamers in Arabidopsis *pdx3* and *sos4* mutant plants and Arabidopsis ecotype Col-0 (WT) determined by HPLC analysis. Values represent the average concentration in plant extracts obtained from two independent sets of plants. Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

Suc, NaCl, and Mannitol Sensitivity

Seedlings of the *pdx3* and *sos4* mutant lines and the wild type were assayed for growth on Murashige and Skoog medium supplemented with 100 mM Suc, NaCl, or mannitol. Addition of Suc to the medium increased root growth in the *pdx3* mutant lines and the wild type (Fig. 6A), with greater increases seen with the *pdx3-1* and *pdx3-2* mutant lines (28% and 24% increases, respectively) as compared with the wild type (14%). By contrast, *sos4* mutant plants were inhibited by Suc, showing 72% reduction in growth compared to growth on medium lacking Suc. Effects of Suc on shoot weight were less dramatic than on root growth. Suc increased shoot weight in *pdx3-1* and *pdx3-2* mutant lines (10% and 24% increases, respectively), but had no effect on the wild type and did not significantly reduce shoot growth of the *sos4* mutant as compared to the wild type (Fig. 6B).

In agreement with the report of Shi et al. (2002), we also found that root growth of the *sos4* mutant plants was strongly inhibited by NaCl (88% reduction; Fig. 6A). Shoot growth of *sos4*, however, was not significantly different from that of the wild type on NaCl (Fig. 6B). NaCl reduced root growth and shoot weight of the *pdx3* and wild-type plants, with the *pdx3-1* and *pdx3-2* mutant lines showing less reduction in root growth (27% and 18% reduction, respectively) and shoot weight (12% and 9% reduction, respectively) than the wild type (32% and 22% reduction, respectively, in root growth and shoot weight). Mannitol reduced root growth and shoot weight in *pdx3* and *sos4* mutants and in the wild type (Fig. 6, A and B). Reductions in root growth were greater than in shoot weight, both in the mutant lines and in the wild type.

Response to High Light, Chilling, and Drought

Eight- to 9-week-old plants of the *pdx3* and *sos4* mutant lines and wild type grown in soil were assayed for their response to high light, chilling, and drought. Plants of all three mutant lines showed a reduction in biomass when grown under control conditions (20°C, 8-h photoperiod at 200 μmol s^{-1} m^{-2}) as compared to the wild type (Fig. 7A). The *sos4* mutant plants showed higher reduction in biomass (50%) than the *pdx3-1* and *pdx3-2* mutant lines (18% and 17%, respectively). All plants (*pdx3* and *sos4* mutant lines and wild type) growing under high light conditions showed dark red pigmentation due to accumulation of anthocyanins (data not shown). However, high light conditions increased growth in wild-type and *sos4* plants, whereas *pdx3* mutant plants did not have increased growth under high light as compared to control conditions (Fig. 7B). An evident reduction in plant size under chilling conditions was observed in the mutant lines and the wild type, which was then reflected in a reduction in dry weight of these plants (Fig. 7B). *sos4* plants were more tolerant to drought than wild-type and *pdx3* plants, a phenotype also seen with plants

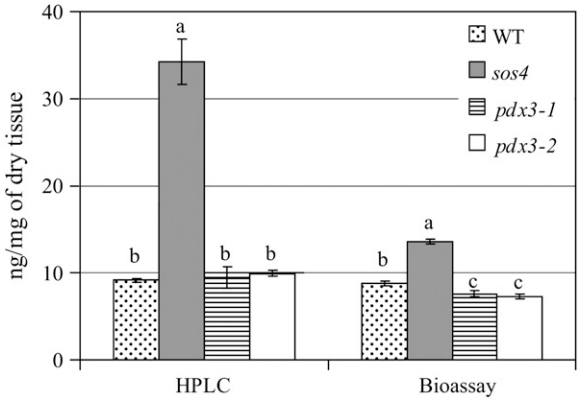

Figure 9. Comparison of total vitamin B_6 in Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT) determined by yeast bioassay and HPLC analysis. HPLC values represent the sum of PMP, PM, PLP, PL, PNP, and PN. Total vitamin B_6 values represent the average concentration in plant extracts obtained from two independent sets of plants. Means followed by the same letter are not significantly different at $P = 0.05$ according to the Waller-Duncan *k*-ratio *t* test.

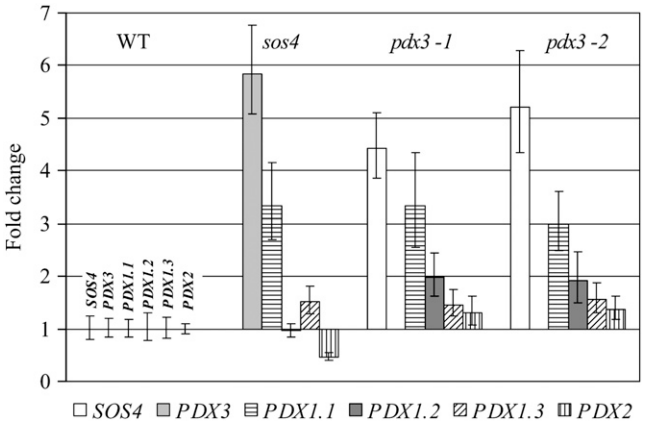

Figure 10. Gene expression of de novo and salvage pathway genes in Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis Col-0 (WT). Lines are indicated across the top of the figure and fold changes in expression level for each gene are shown by the bars. Data represent the average of three replications. Genes were amplified by qRT-PCR using the primers listed in Table II and gene expression was normalized to *UBQ10* expression.

mutant in the de novo pathway gene *PDX1.3* (Supplemental Fig. S1).

Analysis of Vitamin B_6 Levels by HPLC Analysis and Yeast Bioassay

The *sos4* mutant showed significant shifts in vitamer concentration compared to the wild type (Fig. 8). Higher levels of PM, PLP, and PN, with a 2-fold increase in PM and PN and an almost 9-fold increase in PLP, were observed in *sos4* mutant plants. The increase in these vitamers was reflected in a 3.7- and a 1.5-fold increase in total vitamin B_6 measured using HPLC analysis and yeast bioassay, respectively (Figs. 8 and 9). The *sos4* mutant also showed 69% reduction in PMP levels compared to the wild type (Fig. 8).

Total vitamin B_6 concentration in *pdx3* mutant lines was significantly lower than in the wild type, measured using yeast bioassay (Fig. 9). Although *pdx3* mutant lines did not show significant changes in total vitamin B_6 concentration as assayed by HPLC, slight shifts were observed in some vitamer levels (Fig. 8).

Regulation of B_6 Pathway Genes in Mutant Lines

Expression of salvage and de novo pathway genes in wild-type and mutant plants was assayed by qRT-PCR. The *pdx3* and *sos4* mutants had increased expression of the other salvage gene (*SOS4* and *PDX3*, respectively; Fig. 10). *PDX3* was up-regulated almost 6-fold in the *sos4* mutant and the expression of *SOS4* was increased 4.4- and 5.2-fold in the *pdx3-1* and *pdx3-2* mutant lines, respectively, as compared to the wild type. Up-regulation of *PDX3* in the *sos4* mutant correlates with the increased PDX3 expression seen in the enzyme assay (Fig. 4). Expression of the de novo pathway *PDX1* genes also was affected in the *pdx3* and *sos4* mutant lines (Fig. 10). *PDX1.1* was the most up-regulated, with 3-fold increases in expression in the *sos4* and *pdx3* mutants. *PDX1.2* was up-regulated 2-fold in the *pdx3* mutants, but not in *sos4. PDX1.3* was up-regulated less than 2-fold in all mutants. Expression of the de novo pathway gene *PDX2* was not dramatically affected, with very slight increases in the *pdx3* mutant lines and down-regulation in the *sos4* mutant (Fig. 10).

PDX1 Activity in Mutant Lines

sos4 and *pdx3* mutants differ significantly in PLP levels. This difference may be explained by increases in the de novo pathway; however, gene regulation studies showed similar expression of the de novo pathway genes. To further investigate this observation, we assayed PDX1 enzyme activity in wild-type and mutant lines using an assay developed for measuring PDX1 activity in tobacco (Herrero and Daub, 2007). In agreement with the PLP levels, but in contrast to gene expression data, the *sos4* mutant showed an increase in PDX1 activity compared to the wild type (Fig. 11), whereas *pdx3* mutant lines showed lower PDX1 activity than the wild type. Thus, PLP levels of the *sos4* and *pdx3* mutants correlate with PDX1 activity, but do not correlate with expression of the *PDX1* genes.

DISCUSSION

Arabidopsis plants mutant in the vitamin B_6 salvage pathway *PDX3* and *SOS4* genes showed phenotypic changes, altered vitamin B_6 vitamer concentrations, and changes in enzyme activity and regulation of salvage and de novo pathway genes. *pdx3* and *sos4*

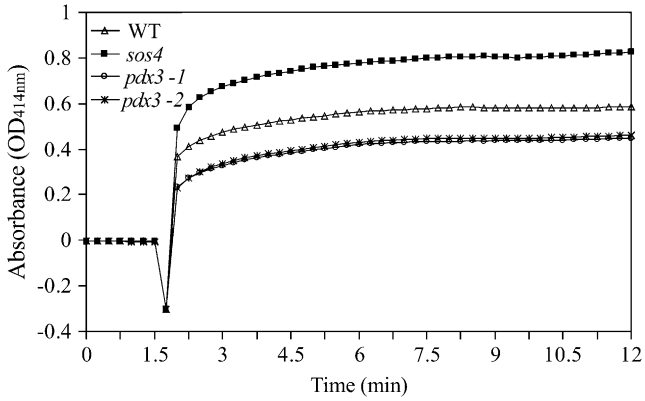

Figure 11. PDX1 activity in Arabidopsis *pdx3* and *sos4* mutant lines and Arabidopsis ecotype Col-0 (WT). The activity of PDX1 was measured in crude extracts of the mutant lines and WT by monitoring the formation of PLP in enzyme reactions containing the same amount of total protein for the mutant lines and WT. Before the addition of the substrate, a 1.5-min baseline was established. Absorbance values represent the average of three replications.

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

Gene	Forward Primer	Reverse Primer
SOS4	5′-CGCTCTGTAAACCCGAATCTTAC-3′	5′-GTTGTATGTGCCTGAAGAACTGG-3′
PDX3	5′-AACTGATCCTACGGTACAGTTTCG-3′	5′-GAGAAACAAATGCTATGGCTTTGT-3′
PDX1.1	5′-TCTCCCTTCTCCGTGAAAGTTG-3′	5′-GCGTTGACGACATCCATGATT-3′
PDX1.2	5′-AGGTCGGATTAGCTCAGGTACTTC-3′	5′-CGGATTCAGCGAGCTTAGCTT-3′
PDX1.3	5′-TTTGCGGTTGCCGGAAT-3′	5′-ATCATCGCCGCACCTTCA-3′
PDX2	5′-GTTCATACGTGCTCCAGCTGT-3′	5′-TTGATGGGACGGGATAATCC-3′
UBQ10	5′-CACACTCCACTTGGTCTTGCG T-3′	5′-TGGTCTTTCCGGTGAGAGTCTTCA-3′
ACTIN2	5′-TCCCTCAGCACATTCCAGCAGAT-3′	5′-AACGATTCCTGGACCTGCCTCATC-3′

mutant lines also showed different in vitro and in vivo responses to stress conditions compared to wild-type plants. PMP/PNP oxidase (PDX3) and PL kinase (SOS4) are key enzymes involved in the vitamin B_6 salvage pathway in Arabidopsis (Shi et al., 2002; Sang et al., 2007), where the nonphosphorylated forms of vitamin B_6 are converted to PLP, the active cofactor of vitamin B_6, essential in many aspects of primary metabolism. Additionally, vitamin B_6 has been linked to stress responses in plants (Shi et al., 2002; Denslow et al., 2005, 2007; Sang et al., 2007) and is essential for root development (Shi and Zhu, 2002; Chen and Xiong, 2005; Titiz et al., 2006). Therefore, it is not surprising that Arabidopsis *pdx3* and *sos4* plants are affected in their growth and development under normal growth and stress conditions.

As previously reported by Shi et al. (2002), Arabidopsis *sos4* plants grown under in vitro conditions show distinct reduction in root growth under normal conditions as well as root hypersensitivity to NaCl, attributed to higher accumulation of Na^+ and lower retention of K^+ compared to wild-type plants. Under their conditions, shoot growth was not affected under normal growth conditions but was reduced on medium containing NaCl. Our results support their root growth findings. However, in our studies, we found that shoot growth of *sos4* mutant plants was reduced under normal conditions, but was not significantly different from the wild type when grown on NaCl. In addition to NaCl sensitivity, *sos4* mutant plants also showed in vitro root hypersensitivity to Suc, as well as chlorosis and a visibly smaller in vivo shoot size when grown under normal conditions, reflected in the reduction of dry weight compared to wild-type plants. Similar changes in phenotype, including chlorosis and root hypersensitivity to Suc, were also observed in experiments conducted in our laboratory with Arabidopsis plants mutant for the *PDX1.3* gene involved in the vitamin B_6 de novo pathway (E. Rueschhoff and M. Daub, unpublished data). Interestingly, *sos4* plants showed resistance to drought compared to wild type. However, plants mutant in the de novo pathway gene *PDX1.3*, which produce only 33% of total B_6 levels of the wild type (E. Rueschhoff and M. Daub, unpublished data), showed the same level of drought tolerance (Supplemental Fig. S1). Thus, drought tolerance is unrelated to total B_6 levels and may be due to the smaller and more compact rosette morphology of both of the *sos4* and *pdx1.3* mutants as compared to rosettes of the *pdx3* mutant lines and the wild type.

Although *pdx3* mutant plants showed a decrease in root and shoot growth and in total vitamin B_6 compared to wild-type plants, these mutant lines were not as debilitated as *sos4* mutant plants. The growth of *pdx3* mutant plants was not affected in the presence of Suc and NaCl. However, *pdx3* mutant plants were incapable of increased growth under high light conditions and showed decreased PDX1 activity as compared to both wild-type and *sos4* mutant plants. The less dramatic phenotype may be due to the fact that these mutants are only reduced in PDX3 activity and are not completely deficient in this enzyme.

Our results demonstrate alterations in total B_6 levels and in levels of the different vitamers in the *sos4* and *pdx3* mutant plants. *pdx3* mutant plants showed significantly lower overall levels of total B_6 when measured by yeast bioassay. Levels of each individual vitamer measured by HPLC, however, were not significantly different from the wild type. By contrast, the level of total B_6 in the *sos4* mutant was significantly greater than the wild type, with significant shifts in levels of individual vitamers. The *sos4* mutant had significantly higher PM and PN levels with a corresponding decrease in PMP. Studies on the activity of the Arabidopsis (Lum et al., 2002) and wheat (Wang et al., 2004) PL kinases, SOS4 and TaPdxK, respectively, have only tested for PL kinase activity. However, Shi et al. (2002) confirmed the activity of SOS4 by complementation of *E. coli* mutants deficient in *pdxK*, which encodes a PL/PN/PM kinase, and concluded that SOS4 is functionally homologous with PdxK. The increased levels in PN and PM showed by *sos4* mutant plants in the HPLC analysis support the hypothesis that SOS4 can also phosphorylate PN and PM, consistent with SOS4 encoding a PL/PN/PM kinase.

An unexpected result of the vitamer analysis was the increase in PLP levels in the *sos4* mutant, a result contrary to predictions. One hypothesis that would explain this observation is increased activity of the de novo pathway. Gene regulation experiments did not support this hypothesis. Both the *pdx3* and *sos4* mutant lines showed similar increased expression of *PDX1.1* and *PDX1.3*, and expression of *PDX1.2* and *PDX2* was actually less in *sos4* as compared to the *pdx3* mutants.

However, PDX1 enzyme activity did correlate with PLP levels, with the *sos4* plants having the highest PDX1 activity followed by wild-type and then the *pdx3* plants. The *sos4* mutant also had higher PDX3 activity than the wild type, indicating greater activity of both salvage and de novo pathways in this mutant. Overall, increased expression of both the salvage and the de novo genes and altered activity of PDX1 and PDX3 in the mutants confirm that plants mutant in PDX3 and SOS4 affect the normal pathway of vitamin B_6 biosynthesis.

The increase in PLP in *sos4* mutant plants suggests the presence of an additional kinase in Arabidopsis that would preferentially phosphorylate PL, hence the increase in PN and PM. In *E. coli*, an alternate kinase (PdxY) is involved in the vitamin B_6 salvage pathway (Yang et al., 1998). This kinase was shown to have specific activity for PL and its function is confined to the salvage pathway. Additionally, in *B. subtilis*, ThiD, a kinase involved in thiamine biosynthesis, was recently shown to have activity for PL, PM, and PN (Park et al., 2004). We have identified two Arabidopsis genes encoding predicted proteins with homology to ThiD and PdxY. At5g58730 is predicted to encode a protein with 27% identity and 48% similarity to *E. coli* PdxY, and At1g22940 (also known as THI1 and involved in thiamine biosynthesis; Machado et al., 1997) encodes a predicted protein with 31% identity and 51% similarity to *B. subtilis* ThiD. Future studies are needed to confirm that these loci encode enzymes involved in the B_6 salvage pathway and to determine whether they are responsible for the increased PLP found in *sos4* mutant plants.

As mentioned before, the PMP/PNP oxidase in *E. coli*, PdxH, is essential for PLP biosynthesis because this enzyme converts the PNP synthesized in the de novo pathway into PLP (Laber et al., 1999) and *pdxH E. coli* mutants are incapable of growth in minimal medium (Lam and Winkler, 1992). In plants, however, PLP is the first vitamer synthesized via the de novo pathway (Tambasco-Studart et al., 2005); thus, mutations in *PDX3* in plants would not be expected to be lethal. Our mutants resulted from insertions in the promoter region of the gene and still retained some enzyme activity; thus, it is not known whether this gene is essential for viability. The *pdx3* mutant lines had approximately half the PDX3 activity of the wild type, resulting in only slightly less total B_6 and no significant differences in levels of individual vitamers. However, these changes had measurable effects on phenotype, including less shoot growth and an inability to increase growth under high light conditions. Gene regulation studies also indicated that reduced PDX3 activity resulted in increased expression of both the de novo and salvage pathway genes, which may have allowed for the near-normal production of B_6.

Although *sos4* mutant plants produce elevated levels of B_6, they show significant reduction in root and shoot growth and increased sensitivity to NaCl and Suc, suggesting that elevated B_6 levels may be debilitating rather than advantageous. This hypothesis is supported by studies in animals and microorganisms, where elevated levels of vitamin B_6 have been shown to be problematic. Large doses of vitamin B_6 can cause sensory neuropathy syndrome in animals (Schaumburg et al., 1983), and overproducing microorganisms excrete excess amounts of vitamin B_6 into the medium (Argoudelis, 1999; Chumnantana et al., 2001) and show delayed recovery from the stationary phase (Rodríguez-Navarro et al., 2002). In our studies to overexpress the B_6 de novo pathway genes in tobacco (Herrero and Daub, 2007), 50% of the transgenic lines constitutively expressing the two de novo genes, *PDX1* and *PDX2*, showed delays in seed germination and early seedling growth, whereas none of the transgenic plants expressing either *PDX1* or *PDX2* alone showed germination and growth delays. These results suggest that elevated B_6 levels may be harmful to cells.

In summary, we have shown that mutations in the Arabidopsis genes encoding PMP/PNP oxidase and PL kinase result in alterations in B_6 levels, growth, and response to stress conditions. Mutations in both loci result in increases in gene expression of both de novo and salvage pathway genes and in activity of the de novo pathway PDX1 enzyme, with enzyme activity but not gene expression correlating with B_6 levels. Our results also suggest the presence of additional genes involved in the salvage pathway in Arabidopsis because vitamer shifts in *sos4* mutants were contrary to predictions based on the function of PL kinase. We are currently conducting experiments to determine the presence of an alternate kinase as well as to identify and characterize a putative PL reductase involved in the vitamin B_6 salvage pathway in Arabidopsis.

MATERIALS AND METHODS

Cloning of the Arabidopsis *PDX3* Gene

To clone *PDX3*, total RNA was extracted from leaf tissue of wild-type Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 plants and reversed transcribed as described previously (Denslow et al., 2005). *PDX3* was amplified from cDNA by PCR using primers 5′-TTTGGGTCATAAAGAGACTACCAATT-3′ and 5′-AATTTGGCCCCATGACTTCTACTCA-3′ and a mix of *Taq* DNA polymerase (Promega) and Pfu Ultra HF DNA polymerase (Stratagene) in a ratio of 10:1. The amplified gene was cloned into vector pCR 4-TOPO and was used to transform *Escherichia coli* One Shot TOP10 chemically competent cells using TOPO TA cloning technology (Invitrogen). Transformants were selected on Luria-Bertani medium containing 50 μg/mL kanamycin. The resulting construct, extracted from the *E. coli* transformants with the Wizard *Plus* Minipreps DNA purification system (Promega), was used to transform freshly prepared *E. coli pdxH*-competent cells of mutants NU877, NU1707, and NU1708, kindly provided by Dr. Malcolm E. Winkler (University of Indiana). These strains are mutant in *pdxH* and are therefore unable to grow on minimal medium lacking PL (Lam and Winkler, 1992). Competent cells were prepared as described previously by Miller and Nickoloff (1995). *E. coli pdxH* mutants were also transformed with vector pCR 4-TOPO containing a control PCR template following the same procedure described above.

Complementation of *pdxH* in *E. coli*

Complementation of *pdxH E. coli* mutants (NU887, NU1707, and NU1708) was determined by comparing the growth of mutants transformed with *PDX3* from Arabidopsis to the growth of the mutants, the parental strain NU816, and the vector-transformed control. Strains were grown overnight at 37°C with

shaking at 250 rpm in 5 mL of liquid Vogel-Bonner 1XE medium (EM) containing 0.01 mM $FeSO_4$ and 1 μM PL. Overnight cultures were centrifuged for 10 min at 1,000 relative centrifugal force (rcf) at 4°C. Pellets were washed twice by resuspending them in 10 mL of sterile deionized water and centrifuging them for 10 min at 1,000 rcf at 4°C. Finally, clean pellets were resuspended in sterile deionized water and the density of the cell suspension was determined using a Beckman DU 600 spectrophotometer at OD_{600}. An aliquot from the cell suspensions containing 5×10^6 bacterial cells was used to inoculate 5 mL of liquid EM plus 0.01 mM $FeSO_4$. Cultures were incubated at 37°C with shaking at 250 rpm and growth was determined at 0, 8, 16, and 24 h by measuring the absorbance at OD_{600}.

Arabidopsis *pdx3* T-DNA Insertion Mutant Lines

Plants from Arabidopsis ecotype Col-0 with T-DNA insertions located in two different sites of the *PDX3* promoter region (SALK_060749 and SALK_149382; Alonso et al., 2003; later named *pdx3-1* and *pdx3-2*, respectively; Fig. 3A) obtained from the ABRC were screened by PCR to verify the T-DNA insertion and identify homozygous mutant plants. Seeds of SALK_060749 and SALK_149382 and the wild type were surface sterilized in a 50% commercial bleach solution (6.15% NaOCl) plus 0.01% Triton X-100 for 10 min and rinsed two to three times with sterile deionized water. Seeds were suspended in 0.1% agarose and plated onto Murashige and Skoog medium (Caisson Laboratories) with 1.2% agar, pH 5.7. Plates were placed at 4°C for 48 h to synchronize germination and then incubated at 22°C and 8-h photoperiod for about 3 weeks. Plants were transferred to Arabidopsis growing medium PM-15-13 AIS mix (Lehle Seeds) and placed at room temperature (approximately 24°C) under an 8-h photoperiod until seeds were collected. Total DNA was extracted using the Quick DNA Prep for PCR protocol described by Weigel and Glazebrook (2002). *PDX3* primers (forward primer, 5′-TCCTCATTTTATCTTAGGATATTC-3′; and reverse primer, 5′-CCACCAAACCATCACCACCAT-3′) designed using the SIGnal T-DNA verification primer design tool, powered by the Genome Express Browser Server (http://signal.salk.edu/tdnaprimers.2.html), were used to amplify an approximate 1,200-bp DNA segment in *PDX3* using the following program: 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1.5 min, and 72°C for 7 min. To confirm the T-DNA insertion in the *PDX3* gene, a DNA segment of about 800 bp was amplified using the *PDX3* reverse primer and the right-border primer of the T-DNA insertion (5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′), following the program described above with a 1-min extension at 72°C.

Phenotypic Analysis of *pdx3* and *sos4* Mutant Lines

Plants of the *pdx3-1* and *pdx3-2* mutant lines, Arabidopsis ecotype Col-0, and the *sos4-1* mutant, previously generated by Shi et al. (2002), were monitored from seed germination to senescence (ready for seed harvest), to determine differences in phenotype, days to first flower buds visible, days to first flower open, and the number of seeds produced per silique. Seeds collected from homozygous *pdx3-1* and *pdx3-2* mutant lines, seeds from the *sos4* mutants (kindly provided by Dr. Jian-Kang Zhu), and seeds from Arabidopsis Col-0 obtained from ABRC were sown into 32-pot plastic trays containing PM-15-13 AIS mix and grown at room temperature (approximately 24°C) under an 8-h photoperiod. After 2 weeks, plants were thinned to one plant per pot and grown until senescence. Thirty-two plants per mutant and wild type were analyzed. Additionally, 138 seeds harvested from the plants were plated on Murashige and Skoog medium with 1.2% agar and pH 5.7 to determine the percentage of seed germination. In vitro root growth and foliar biomass were also determined. Seeds of the mutant lines and wild type were surface sterilized and plated on square plates containing Murashige and Skoog medium as described above for 6 d. Plates were oriented vertically for in vitro assays. Six 6-d-old seedlings of each mutant and the wild type were transferred to new square plates containing the same medium, and root length was measured using a ruler every other day for 15 d. All plates were run in triplicate and the experiment was repeated one time.

Stress Responses

The in vitro sensitivity of the *pdx3* and *sos4* mutants and wild type to Suc, mannitol, and NaCl was tested based on a previously described method with some modifications (Wu et al., 1996). Seeds were surface sterilized as above and plated on square plates containing Murashige and Skoog medium with 1.2% agar and pH 5.7. Plates were placed at 4°C for 48 h to synchronize germination and then incubated vertically at 22°C under an 8-h photoperiod. To test Suc, mannitol, and NaCl sensitivity, six 6-d-old seedlings were transferred to square plates containing the same medium alone or supplemented with 100 mM Suc, mannitol, or NaCl. Three replicate plates were used for each treatment and assay plates were oriented vertically. Root length was measured with a ruler every other day for 15 d and the experiment was repeated one time. Response of the mutant lines and wild type to high light, chilling, and drought was tested in growth chambers following a protocol described by Denslow et al. (2007). Plants mutant in the *PDX1.3* de novo gene were also included in the drought experiment as a comparison; these plants produce 33% of the total B6 levels of wild-type plants (E. Rueschhoff and M. Daub, unpublished data). For these experiments, 3-week-old seedlings of each mutant and the wild type were transferred to 32-pot plastic trays containing PM-15-13 AIS mix. The plants to be tested for high light and chilling were randomly arranged in a controlled-environment growth chamber at 20°C under an 8-h photoperiod at 200 μmol s^{-1} m^{-2} light (control chamber). After 3 weeks, one-third of the plants were moved to a growth chamber at 20°C and an 8-h photoperiod at 1,000 μmol s^{-1} m^{-2} light (high light conditions), and another one-third to a growth chamber at 5°C and an 8-h photoperiod at 200 μmol s^{-1} m^{-2} light (chilling conditions). The remaining one-third of the plants was kept in the control chamber. Plants were monitored for phenotypic changes for 2 weeks, after which all plants were harvested and dry weights were determined. The experiment was repeated one time. For the drought experiment, 32 plants of each mutant line and the wild type were randomly arranged in a growth chamber at 20°C and watered once a week for 3 weeks. After this period, watering was stopped in one-half of the plants, and all plants were monitored for phenotypic changes for the next 3 weeks.

Extraction of Vitamin B_6

Vitamin B_6 was extracted from leaf tissue from *pdx3-1*, *pdx3-2*, and *sos4* mutant lines and Arabidopsis ecotype Col-0 obtained from two independent sets of plants. Seeds were sown into 48-pot plastic trays containing PM-15-13 AIS mix, randomly arranged, and grown at room temperature (approximately 24°C) under an 8-h photoperiod. After 2 weeks, plants were thinned to one plant per pot and grown for 6 to 8 more weeks. Leaf tissue from all plants of each mutant and wild type was bulked, lyophilized, and ground in liquid nitrogen. The finely ground tissue was divided into three samples and vitamin B_6 was extracted by adding 8 mL/g of ground tissue of 5% (w/v) TCA (HPLC grade; LabChem). Samples were homogenized with a Sorvall omni-mixer (Du Pont) in a 50-mL stainless steel chamber for 5 min at power setting 5. Homogenates were centrifuged for 15 min at 10,000 rcf at 4°C. Supernatant was transferred to a clean tube and clarified by centrifugation as above. Extracts were kept on ice and in darkness at all times. Leaf extracts were stored in the dark at −20°C until use.

Yeast Bioassay

Levels of total vitamin B_6 in the leaf extracts were determined by a yeast bioassay that quantifies growth of a yeast strain (ATCC 9080) auxotrophic for vitamin B_6. Leaf extracts were prepared using a modified protocol from Denslow et al. (2005). Concentrated leaf extracts were diluted 4-fold with sterile deionized water, and pH was adjusted to 4.8 with sodium acetate. To deglycosylate and dephosphorylate vitamin B_6 vitamers, freshly prepared β-glycosidase (5 mg in 1 mL of water) and acid phosphatase (75 mg in 2 mL of water) were added to 50-mL tubes containing the diluted plant extracts. Tubes were incubated overnight at 37°C with shaking at 70 rpm. Treated leaf extracts were filter sterilized using a 50-mL steriflip unit (0.22 μM; Millipore) and stored in the dark at −20°C until use. Yeast cells were grown on malt agar medium overnight at 30°C. Cells were washed twice with 10 mL of sterile deionized water and pelleted by centrifuging at 4°C and 1,000 rcf for 10 min. Pellets were resuspended in 2 mL of sterile deionized water, and the concentration of the cell suspension was determined using a hemacytometer. For the assay, 14-mL polystyrene tubes containing 5 mL of yeast extract mannitol medium (Becton Dickinson) were inoculated with 5×10^6 yeast cells and 8 μL of treated leaf extract. Standards contained 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 ng of total PN per 5-mL volume. All samples and standards were run in triplicate. Tubes were incubated at 30°C with shaking at 250 rpm. Growth was measured at 17 and 24 h with a spectrophotometer at OD_{540}. Total vitamin B_6 in the leaf extracts was determined based on comparison to the standard curve.

HPLC Analysis

Levels of all six vitamin B_6 vitamers (PN, PM, PL, PNP, PMP, and PLP) were determined by HPLC. A reverse-phase HPLC method described by Valls et al. (2001) was used with some modifications. The HPLC system consisted of a Waters 616 pump, a Rheodyne 7125 injector equipped with a 100-μL sample loop, a Shimadzu RF-10AXL fluorescence detector, and a Waters 600S controller. The chromatographic column was a 250- × 4.6-mm i.d. HyperClone 5-μm BDS C18 (Phenomenex) equipped with a 4- × 3-mm i.d. SecurityGuard C18 (ODS; Phenomenex) guard column. The guard and analytical column were mounted in a column heater set at 35°C. The mobile phase consisted of two components: solution A (0.05 M phosphoric acid in water) and solution B (solution A containing 1% acetonitrile). Both solutions were adjusted to pH 3.2 with 10 N potassium hydroxide prior to and after degassing. Separation was carried out using a mixture of 50% solution A and 50% solution B at a flow rate of 1 mL/min. The detector excitation and emission wavelengths were set at 290 and 395 nm, respectively. Reference standards of PN (PN hydrochloride), PL (PL hydrochloride; Sigma grade), PM (PM dihydrochloride, 98% purity), PMP (98%), and PLP (98%) were obtained from Sigma-Aldrich. PNP was kindly provided by Dr. Christos Argoudelis (University of Illinois). HPLC-grade phosphoric acid, acetonitrile, and water were from Fisher Scientific. Stock solutions of 100 ng/μL of all standards were stored at 4°C and in darkness. Spiking of the leaf extracts with reference standards of PN, PNP, PM, PMP, PL, and PLP was used to confirm peak identity. Six-point standard curves were prepared and run daily before running the leaf extract samples, by injection of standards in the following concentration ranges: PN, 2.5 to 49.1 pmol; PNP, 2.1 to 33.2 pmol; PL, 0.3 to 9.8 pmol; PMP, 4.0 to 80.6 pmol; PM, 0.3 to 8.3 pmol; and PLP, 12.1 to 809.3 pmol. Concentrated leaf extracts were filter sterilized using a 50-mL steriflip unit (0.22 μM; Millipore) and diluted 4-fold with water (HPLC grade) before injection. Concentrations of vitamin B_6 vitamers were determined based on comparison to the standard curve, which was obtained by fitting a linear curve to the average peak area of the different known concentrations for each vitamer. To prevent changes in retention time due to changes in retention caused by the use of a 99% aqueous mobile phase, at the beginning of each day and after every leaf extract injection, the column was flushed with acetonitrile using a solvent gradient starting with 50% A and 50% B and moving to 15% A, 15% B, and 70% acetonitrile for 40 min, followed by 10 min with 15% A, 15% B, and 70% acetonitrile, returning to 50% A and 50% B in a linear gradient for 20 min, and finally 10 min with 50% A and 50% B.

PDX1 and *PDX3* Enzyme Assays

PDX1 activity was measured in crude extracts of *pdx3-1*, *pdx3-2*, and *sos4* mutant lines and Arabidopsis ecotype Col-0 based on an enzyme assay previously described by Herrero and Daub (2007) with some modifications. Crude extracts were obtained by grinding 100 mg of leaf tissue in 400 μL of 50 mM Tris-HCl, pH 8.0. PDX1 activity was determined by monitoring the formation of PLP in a 100-μL reaction containing 80 to 90 μg of total protein from the crude extracts, 25 mM NH_4SO_2, and 10 mM glyceraldehyde-3-P. Before the addition of glyceraldehyde-3-P, a 1.5-min baseline was established in the presence of the crude extract and NH_4SO_2. The reaction progress was monitored spectrophotometrically at 414 nm. The activity of PDX3 was also measured in crude extracts of *pdx3-1*, *pdx3-2*, and *sos4* mutant lines and Arabidopsis ecotype Col-0 with a colorimetric procedure previously described for *E. coli* (Zhao and Winkler, 1995) with some modifications. Crude extracts were obtained from 500 mg of lyophilized tissue (obtained from the same samples used for the HPLC analysis) homogenized in 1 mL of 200 mM Tris-HCl and 200 mM KPO_4, pH 8.5, with a Silamat S5 (Ivoclar Vivadent) for 20 s. PMP oxidase activity in 1 to 7 mg of total protein from the crude extracts was determined in 0.5-mL reaction mixtures containing 200 mM Tris-HCl, 200 mM KPO_4, pH 8.5, and 0.2 mM PMP. After 1 h of incubation at 37°C, reactions were stopped by addition of 50 μL of chilled 50% (w/v) TCA. Precipitated protein was pelleted by centrifuging at 4°C and 1,500 rcf for 10 min, and supernatant transferred to a clean tube. PLP formation was measured following addition of 2% (w/v) phenylhydrazine in 10 N H_2SO_4 and incubation on ice for 30 min (Wada and Snell, 1961) by determining the absorbance at OD_{410}. PDX3 activity data were analyzed with a regression line adjusted to the absorbance values obtained for increasing amounts of total protein in the enzyme reactions conducted for each mutant and the wild type. Total protein concentration in the crude extracts was determined with the Bradford protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as the standard, following the protocol recommended by the manufacturer. All enzyme reactions were repeated three times.

qRT-PCR

Expression of the de novo pathway genes *PDX1.1*, *PDX1.2*, *PDX1.3*, and *PDX2* (Denslow et al., 2007) and the salvage genes *SOS4* and *PDX3* was determined in *pdx3-1*, *pdx3-2*, and *sos4* mutant lines and Arabidopsis ecotype Col-0 using qRT-PCR. Total RNA was extracted from leaf tissue and reverse transcribed as described previously (Denslow et al., 2005). The qRT-PCR reactions consisted of 2 μL of cDNA, reverse transcribed from 1 μg of RNA, 5 μL of 2× SYBR Green master mix (Applied Biosystems), and gene-specific primers at a concentration of 0.9 pmol/μL (Table II). All primers were designed using Primer Express (Applied Biosystems) and cDNA sequences were obtained from The Arabidopsis Information Resource. Reactions were adjusted to a total volume of 10 μL with sterile deionized water. All genes were compared to expression of the genes encoding *UBQ10* and *ACTIN2*. The primers used to amplify these genes were at a concentration of 300 nM and are shown in Table II. All reactions were run in triplicate on a DNA Engine Opticon 2 System (Bio-Rad Laboratories) using the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and the experiment was repeated three times. Only gene expression data normalized to the *UBQ10* gene are shown as these data were the most constant among repetitions.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM124376 (Arabidopsis *PDX3*), M92351 (*E. coli pdxH*), and AF400125 (Arabidopsis *SOS4*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Response of Arabidopsis *sos4* and *pdx1.3* mutant lines and Arabidopsis ecotype Col-0 (wild type) to drought conditions.

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