Investigation of PDX1 and its role in a novel vitamin B6 biosynthesis pathway in Arabidopsis thaliana

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INVESTIGATION OF *PDX1* AND ITS ROLE IN A NOVEL VITAMIN B6 BIOSYNTHESIS PATHWAY IN *ARABIDOPSIS THALIANA*

A dissertation submitted to

ETH ZURICH

for the degree of

DOCTOR OF SCIENCES

presented by

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17th February 1973

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2008
“The significant problems we face cannot be solved at the same level of thinking we were at when we created them”

Albert Einstein
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SUMMARY

Vitamin B6 is an essential metabolite in all organisms. It can act as a coenzyme for numerous metabolic enzymes and has recently been shown to be a potent antioxidant. Plants and microorganisms have a de novo biosynthetic pathway for vitamin B6, but studies of its biosynthesis have been mainly restricted to Escherichia coli. Recently, based on genetic evidence in fungi, it appears that a novel pathway, distinct from the well-elucidated pathway in E. coli, is present, which involves two genes (PDX1 and PDX2) neither of which is homologous to any of those involved in the E. coli pathway. Furthermore, it seems that only a small subset of eubacteria utilize the “E. coli” type pathway while all other vitamin B6 synthesizing organisms use the alternative pathway. In this study, a biochemical and physiological characterization of PDX1 was performed in the model plant Arabidopsis thaliana. Three homologues of PDX1 were identified, namely PDX1.1, PDX1.2 and PDX1.3, of which only PDX1.1 and PDX1.3 appear to be functional in pyridoxal 5'-phosphate (PLP) biosynthesis. Reconstitution of vitamin B6 biosynthesis could be accomplished in vitro in the presence of an ammonium source and either of the two functional PDX1 proteins and the substrates ribose 5-phosphate or ribulose 5-phosphate and glyceraldehyde 3-phosphate or dihydroxyacetone phosphate. Functional complementation of a yeast vitamin B6 auxotrophic mutant backed up these findings, and demonstrated that PDX1.1 and PDX1.3, but not PDX1.2 is functional in PLP biosynthesis. Development of single pdx1.1 and pdx1.3 mutants was affected; the disruption of PDX1.3 resulted in severe retardation of root growth, whereas disruption of PDX1.1 also affected root growth, but not as strongly. Disruption of both genes causes embryo lethality at the globular stage. Detailed examination of the single mutants, in addition to those that only have a single functional copy of either gene, indicates that although these genes are partially redundant in vitamin B6 synthesis, PDX1.3 is more requisite than PDX1.1. Developmental distinctions correlate with the vitamin B6 content. Furthermore, evidence is provided that in addition to being essential for plant growth and development, vitamin B6 also plays a role in stress tolerance and photoprotection of plants. Certain cis acting elements could be identified in the promoters of the functional PDX1.3 and PDX1.1 genes, and the unique presence of the putative auxin and ethylene responsive elements in the PDX1.3 promoter indicates a differential regulation pattern. The observation of a phenotype in pdx1.1 and pdx1.3 etiolated seedlings with discrepancies in the formation of the apical hypocotyl hook suggest a possible role of ethylene. Indeed, ethylene biosynthesis measurements lead to the conclusion that the pdx1.3 mutant is more retarded in biosynthesis of this hormone than the pdx1.1 mutant.
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wirkende Elemente konnten in den Promotoren der funktionellen PDX1.3 und PDX1.1 Gene identifiziert werden, und das Vorkommen von vermutlich Auxin- und Ethylen-spezifischen Sequenzen ausschließlich im PDX1.3 Promotor weist auf ein differentielles Regulationsmuster hin. Die Beobachtung, dass die pdx1.1 und pdx1.3 Mutanten einen Phänotyp aufweisen, in dem die Bildung des apikalen Hypokotylhakens beeinträchtigt ist, lässt eine mögliche Beteiligung des Ethylens vermuten. In der Tat ergaben Messungen der Ethylenbildung, dass diese in der pdx1.3 Mutante stärker reduziert ist als in der pdx1.1 Mutante.
1 General introduction

The discovery of vitamin B6

In the 1930s, after the discovery of the vitamins thiamine, riboflavin, niacin and pantothenic acid, scientists began to search for what they assumed was a missing member of the B family, that is, a vitamin that could cure acrodynia, a skin disorder in rats that appeared to resemble the human disease, pellagra. Pellagra was already known to be a deficiency disease that could be prevented or cured by adding niacin (vitamin B₃) to the diet. As acrodynia was a pellagra-like dermatitis in rats, logically then, niacin should work on acrodynia as well. But in 1934, Paul György, a Hungarian-born physician who had emigrated to the United States, discovered that it did not work at all. Moreover, György had learned earlier that neither vitamin B₁ (thiamine) nor vitamin B₂ (riboflavin) had much effect on the rat pellagra either (György, 1934). A few years later, in 1938, five separate groups of researchers including György with his several coworkers, and Richard Kuhn and his collaborators in Heidelberg finally isolated a crystalline material from yeast that, among other things, could both prevent and cure acrodynia (Lepkovsky, 1979). Duly named vitamin B₆ (or pyridoxine), the new vitamin's structure was determined in 1939 and, in that same year, its synthesis was accomplished by S. A. Harris and Karl Folker, who were industrial chemists of the time (Harris and Folkers, 1939)

The vitamin was named pyridoxine to indicate its structural homology to pyridine (Figure 1). Later it was shown that vitamin B₆ could exist in other, slightly different, chemical forms, termed pyridoxal and pyridoxamine. The B₆ vitamers differ by a variable group at the 4’ position which can either be a hydroxyl (pyridoxol, PN), an aldehyde (pyridoxal, PL), or an amino (pyridoxamine, PM) group (Figure 1). All three forms of the vitamin can act as precursors of the active forms of vitamin B₆; the phosphorylated forms, pyridoxal 5’-phosphate (PLP) and pyridoxamine 5’-phosphate (PMP) are active as enzyme cofactors (Figure 1).
**Figure 1. Vitamers of vitamin B6**

**PLP-dependent enzymes**

PLP and PMP play a vital role as the cofactor of a large number of essential enzymes in all living organisms. Since its discovery in the 1930’s, vitamin B6 is well known in the medical field as being involved in more bodily functions than any other single nutrient and that it is required for the maintenance of physical as well as mental health (Gengenbacher et al., 2006). Enzymes dependent on PLP as a cofactor catalyze a wide variety of chemical reactions mainly involving amino acids. The reactions carried out by PLP-dependent enzymes include the transfer of an amino group, decarboxylation, racemization, and β- or α-elimination or replacement. Such versatility arises from the ability of PLP to covalently bind the substrate, and then to act as an electrophilic catalyst, thereby stabilizing different types of carbanionic reaction intermediates (Drewke and Leistner, 2001). PLP-dependent enzymes belong to a small number of evolutionary lineages, but encompass more than 140 distinct catalytic functions, thus representing a remarkable example of divergent evolution (B6-database, 2006). As shown in Figure 2, the different colour sections correspond to the indicated enzyme families, which are then divided into several subcategories according to their specific enzymatic activity. Some significant functions of PLP include acting as a cofactor in transamination, decarboxylation, and α,β-elimination reactions of amino acid metabolism (Drewke and Leistner, 2001; Mittenhuber, 2001), in addition to the synthesis of precursors to antibiotics. Besides these roles, PLP also represents...
an important cofactor for the degradation of lipids and storage carbohydrates, such as glycogen (Friedrich, 1988; Combs, 1998).

![Figure 2. Enzymes with their activities indicated as EC numbers (the numbers shown in the rings) which contain PLP as a cofactor: the rings represent the evolutionary lineages, during the course of evolution, as the size of the rings get larger, the segments that it is divided into increases, meaning that each subgroup or family divides into new subgroups, which have new additional enzymatic specificities (B6-database, 2006).](image)

Apart from its function as a cofactor, vitamin B6 is also thought to act as a protective agent against reactive oxygen species, such as singlet oxygen (Bilski et al., 2000; Chen and Xiong, 2005).

**The biosynthesis of vitamin B6**

Two types of metabolic pathways for PLP synthesis are known: the *de novo* pathway and the salvage pathway. Most unicellular organisms and plants can obtain the active form of vitamin B6 using one of these pathways (Tanaka et al., 2005).
1.0.1 Vitamin B6 biosynthesis in *Escherichia coli*

De novo vitamin B6 biosynthesis has been extensively studied in the last decades, but predominantly in the gram-negative model organism, *Escherichia coli* (Spenser and Hill, 1995; Hill et al., 1996; Cane et al., 1998; Laber et al., 1999; Banks and Cane, 2004).

From molecular cloning and characterization of genes coding for enzymes involved in vitamin B6 biosynthesis in *E. coli*, it was shown that the B6 vitamer, PNP, is synthesized by the action of the PdxA and PdxJ enzymes using 4-phosphohydroxy-L-threonine (4PHT) and 1-deoxy-D-xylulose 5-phosphate (DXP) as substrates (Figure 3) (Lam and Winkler, 1992; Zhao and Winkler, 1996; Cane et al., 1998; Laber et al., 1999). Once PNP is synthesized, it can subsequently enter the salvage pathway, such that it can be oxidized by the action of the oxidase, PdxH, to PLP, the active form of the vitamin (Notheis et al., 1995; Zhao and Winkler, 1996). DXP, which is also a precursor in isoprenoid and thiamine biosynthesis, is synthesized by the action of the DXP-synthase (DXS) (Sprenger et al., 1997; Lois et al., 1998) from pyruvate and D-glyceraldehyde-3-phosphate. The other intermediate, 4PHT, is formed through a series of reactions which involve two oxidation and a transamination step starting from D-erythrose-4-phosphate (E4P). In the first oxidation step, E4P is converted to 4-phosphoerythronate (4PE) by the action of glyceraldehyde 3-phosphate dehydrogenase A, GapA (Yang et al., 1998). 4PE is further oxidized by the dehydrogenase, PdxB, to 3-hydroxy-4-phosphohydroxy-α-ketobutyrate (Lam and Winkler, 1992). In a final step, this compound is then transaminated to 4PHT by the enzyme PdxF (SerC), a transaminase which uses glutamate as donor of the amino group and requires PLP as a cofactor for its enzymatic activity (Drewke et al., 1996) (Figure 3). From the six *E. coli* genes described in the above text, two i.e. GapA/B and PdxF, are involved in other metabolic processes, but PdxA, PdxB, PdxJ and PdxH are unique to vitamin B6 biosynthesis. A so called PDX box, a conserved sequence consisting of, ACGT(G/T)AAAATCC, which is sometimes found as a direct repeat, is found upstream from the translation start site of PdxA, PdxB and PdxJ, this may indicate a common regulatory mechanism in PNP biosynthesis for the three mentioned genes (Lam et al., 1992).
Figure 3. Vitamin B6 biosynthesis in *E. coli* (Lam et al., 1992; Zhao and Winkler, 1996; Cane et al., 1998; Laber et al., 1999)
1.0.2 The salvage pathway

B6 vitamers can be converted into one another via a salvage pathway. It was shown by two different groups (Hill et al., 1996; Yang et al., 1996; Yang et al., 1998) that PLP, PNP and PMP can be synthesized by the action of kinases which phosphorylate PL, PN and PM to their respective 5'-phosphates (Figure 4). So far two different kinases exhibiting different substrate specificities have been identified in *E. coli*, the specific PL kinase, PdxY (Yang et al., 1998) and the PL/PN/PM kinase, PdxK (Yang et al., 1996). PMP and PNP can be oxidized to PLP by the action of the oxidase, PdxH (Zhao and Winkler, 1996), or PMP can also be converted to PLP by the action of unspecific transaminases (Figure 4), also a pyridoxal reductase has been identified in yeast (Guirard and Snell, 1988; Nakano et al., 1999). It has been reported that in mammalian cells, which do not possess the *de novo* pathway of vitamin B6 biosynthesis, a similar salvage pathway involving kinases and oxidases exists (Choi et al., 1987; Hanna et al., 1997; Li et al., 2002; Cheung et al., 2003).
Figure 4. The salvage pathway for PLP biosynthesis as in *E. coli* (Yang et al., 1996, 1998)
Despite the nutritional importance of vitamin B6, most of the research on its biosynthesis for several decades had been mainly restricted to *E. coli*. In contrast to detailed knowledge concerning vitamin B6 biosynthesis in *E. coli*, until recently not much was known about vitamin B6 biosynthesis in any other organism. There was only one early report for the Gram-positive organism *Bacillus subtilis*, where in order to identify genes whose products might participate in vitamin B6 biosynthesis in this organism, BLAST searches were performed in the “SubtiList” database (Moszer et al., 1995; Moszer, 1998) using the sequences of the *E. coli* vitamin B6 biosynthetic enzymes PdxA/J as queries. The known *E. coli* PdxA/PdxJ sequences were found not to be present in the *B. subtilis* genome. As far as eukaryotic organisms are concerned, plants are known to have high concentrations of the vitamin (Friedrich, 1988), but research on its biosynthesis remained preliminary. In yeast, in 1995, it was discovered from labelling studies (Tazuya et al., 1995), that the origin of the nitrogen atom of pyridoxine differed in *Saccharomyces cerevisiae*. In *S. cerevisiae*, nitrogen originating from glutamine rather than glutamate, as is the case in *E. coli* (Tazuya et al., 1995), is incorporated into pyridoxine. Based on the facts that Pdx A/J could not be found in the SubtiList database, and that the origin of the nitrogen atom in yeast differed, it was thought that in these organisms another pathway of vitamin B6 biosynthesis might be operational and that the *E. coli* pathway may not be found in all organisms.

1.0.3.1 Discovery of the second *de novo* pathway

*Cercospora nicotianae* is a filamentous fungus that is resistant to cercosporin, a light activated singlet oxygen generating toxin it produces in culture during plant parasitism. To study the specific genes and proteins involved in the organism’s own resistance to cercosporin, Margaret Daub’s group, through mutant complementation studies and the generation of singlet oxygen sensitive SOR1 null mutants by targeted gene replacement, suggested that SOR1 plays a role in *C. nicotianae* resistance to both cercosporin and other photosensitizers (Ehrenshaft et al., 1998; Ehrenshaft et al., 1999b). Surprisingly even though singlet oxygen resistance is uncommon, SOR1 homologs were found to be widespread, occurring in numerous organisms within the four kingdoms of life (Ehrenshaft et al., 1998). In addition to having a widespread distribution, SOR1 is one of the most highly conserved proteins identified to date (Figure 5) (Braun et al., 1996; Galperin and Koonin, 1997). These results implicated that the SOR1 protein is involved in a conserved yet unknown metabolic function. This mystery was unravelled in 1999 with the work of Margaret Daub and her co-workers when they discovered a second phenotype.
for SOR1 that explains its strong conservation among diverse organisms. They showed that SOR1 is necessary for the synthesis of vitamin B6 in *C. nicotianae*. In this study, six *sor1* mutant strains of *C. nicotianae* were tested, three of which were generated by UV mutagenesis and three null strains that were generated by targeted gene replacement. All of the six strains required pyridoxine for growth, when they were tested on minimal medium (Figure 6) and all of the same mutant strains were restored to prototrophy when transformed with the *SOR1* gene. As reported in the same study, it was by chance discovered that the PN auxotrophic strain of *Aspergillus flavus*, became prototrophic when transformed with *SOR1* from *C. nicotianae*. In the same year, Osmani et al. (1999) reported that mutations in the SOR1 homolog, PyroA, of the filamentous fungus *Aspergillus nidulans* led to vitamin B6 auxotrophy.

The singlet oxygen resistance protein SOR1 of *C. nicotianae*, later named “PDX1” for pyridoxine biosynthesis, is classified as a member of the UPF0019 or SNZ (SNooZe) family in yeast and has been previously identified as a stationary phase inducible protein in these organisms, an ethylene responsive protein in plants (Sivasubramaniam et al., 1995), and a hydrogen peroxide inducible protein in *Bacillus subtilis* (Mittenhuber, 2001). More recently, it has been confirmed through genetic studies that the *Neurospora crassa* Pdx1, *S. cerevisiae* SNZ1 and *B. subtilis* YaaD, which are the respective PDX1 homologs, are essential for vitamin B6 biosynthesis in these organisms (Bean et al., 2001; Rodriguez-Navarro et al., 2002; Sakai et al., 2002; Burns et al., 2005; Raschle et al., 2005).
Figure 5. Amino acid alignment of PDX1, from the following organisms: Arabidopsis thaliana, Oryza sativa, Ginkgo biloba, Cercospora nicotianae, Emericella nidulans, Saccharomyces cerevisiae, Plasmodium falciparum, Plasmodium yoelli, Bacillus subtilis, Thermotoga maritima and Pyrococcus horikoshii.
Phylogeny of vitamin B6 biosynthetic enzymes

An extensive phylogenetic analysis on the distribution of the Pdx genes has been performed by G. Mittenhuber in 2001 and is summarized here. According to the classification made by C.R. Woese (Woese et al., 1990), on 16S/18S ribosomal RNA derived phylogenetic trees, the first tree (Figure 7, top) comprises organisms belonging mostly to the ‘γ’ subdivision of proteobacteria in which the PdxA/PdxJ genes are responsible for vitamin B6 biosynthesis. The second tree contains organisms in which the alternative pathway is in operation (Figure 7, bottom). In this tree, a clear classification of the organisms into the three domains of life can be observed (Mittenhuber, 2001) (The data set which was used for the construction of the trees is included in the appendix I). The most striking result from this phylogenetic analysis is that not a single organism has been identified to date that contains genes of both pathways responsible for vitamin B6 biosynthesis. In other words, these two pathways are mutually exclusive, and organisms either have the E.coli PdxA/J genes, or the alternative pathway, PDX1/2 genes, but not both. Secondly, the alternative de novo pathway genes are much more widespread, among diverse taxa of organisms (Table 1).
Figure 7. Unrooted phylogenetic trees based on 16S rRNA sequences. Taken from Mittenhuber, 2001. The tree on the top has been constructed from sequences from all organisms in which a PdxA (and/or a PdxJ gene) was detected; the tree at the bottom has been constructed from the sequences from all organisms in which a PDX1 like gene was detected. The orange, blue and pink colored circles, show clustering into bacteria, eukarya and archae, respectively (see appendix I, for the list of the abbreviations of the organisms which were included in the dataset used for construction of the trees).
<table>
<thead>
<tr>
<th>Plant</th>
<th>PDX1/2</th>
<th>PdxA/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hevea brasiliensis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Brassica napus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Stellaria longipes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Archaea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrococcus furiosis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanococcus jannaschii</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>+</td>
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<tr>
<td>Archaeoglobus fulgidus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Eubacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thermotoga maritima</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Deinococcus radiodurans</td>
<td>+</td>
<td>-</td>
</tr>
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<td>Mycobacterium tuberculosis</td>
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<td>Clostridium acetobutylicum</td>
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<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>+</td>
</tr>
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<td>Helicobacter pylori</td>
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<td>Yersinia pestis</td>
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<td>+</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. List of organisms which have the PDXA/J or the PDX1/2 gene homologs taken from Ehrenschaft et al. (1999a)
Functionality of PDX1 and PDX2

A second highly conserved gene family named “UPF0030 or SNO” in yeast and YaaE in *B. subtilis* which is now referred to as PDX2, is found in the same organisms that have a PDX1 homolog (Galperin and Koonin, 1997). PDX2 normally appears to be located in close proximity to PDX1. In fact in *B. subtilis*, PDX1 and PDX2 are part of a two gene operon. Furthermore, *S. cerevisiae* PDX1 and PDX2 have been shown to interact in a two-hybrid assay and their expression seems to be co-regulated (Padilla et al., 1998). More recently it has been demonstrated in *B. subtilis* and *C. nicotianae* that PDX2 is also essential for pyridoxine biosynthesis (Ehrenshaft and Daub, 2001; Sakai et al., 2002).

As mentioned above, in *E. coli*, PdxA and PdxJ are required for the formation of the pyridine ring of pyridoxine 5’-phosphate. Specifically, PdxA is proposed to catalyze the oxidation and decarboxylation of 4-phosphohydroxy-L-threonine; whereas PdxJ completes the condensation and ring closure reactions (Cane et al., 1998; Laber et al., 1999). At the onset of this thesis in 2004, it was not known how PDX1 and PDX2 produce vitamin B6, or which vitameric form of the vitamin is made. Based on two-hybrid analyses in yeast, it was proposed that the PDX1 and PDX2 proteins interact and form a complex (Padilla et al., 1998). This prediction was thereafter confirmed by Dong et al., (2004) who showed by affinity chromatography that the yeast proteins indeed form a complex. Furthermore, amino acid sequence alignment studies had indicated that PDX2 was likely to be the glutaminase domain of a class 1 glutamine amidotransferase (Galperin and Koonin, 1997; Mittenhuber, 2001). Kondo and his co-workers (Dong et al., 2004) expressed the *S. cerevisiae* PDX2 in *E. coli* and indeed found glutamine hydrolyzing activity for PDX2. However, glutaminase activity was only observed in the presence of PDX1. In general, glutamine amidotransferases consist of two domains, a glutaminase domain, which hydrolyzes glutamine to glutamate and ammonia, and a synthase domain that accepts and utilizes the ammonia released from the glutaminase domain (Zalkin and Smith, 1998). It was, therefore, postulated that PDX1 is likely to be the synthase subunit of the glutamine amidotransferase hetero-oligomeric complex (Belitsky, 2004; Dong et al., 2004). However, the substrates of PDX1 were not known. It was later discovered from labeling studies in fungi that the pathway takes a different chemical route where the C5 unit of the pyridoxine ring of vitamin B6 is derived from a pentose other than DXP, as in *E. coli* (Gupta et al., 2001; Kondo et al., 2004; Burns et al., 2005). However, the exact nature of the substrates remained to be determined.
**Vitamin B6 and oxidative stress**

Gene regulation studies in numerous organisms have linked vitamin B6 to oxidative stress. For example, in the bacterium *Bacillus subtilis*, the expression of *Pdx1* is enhanced during treatment with the superoxide generator paraquat and hydrogen peroxide (Antelmann et al., 1997), and in *Saccharomyces cerevisiae*, transcript and protein of one of the three sets of PDX1, SNZ1 accumulate at entry into stationary phase, a time of high oxidative stress (Braun et al., 1996; Padilla et al., 1998).

Although the antioxidant properties of many compounds, such as vitamins C and E and ß-carotene are well known, vitamin B6 was previously not recognized to have antioxidant properties. In the same study in which the alternative pathway was discovered, it was also shown that pyridoxine can quench singlet oxygen *in vitro* at a rate comparable to that of vitamin C and E (Ehrenshaft et al., 1999a). Additionally, the same year, it was shown that in *A. nidulans*, mutations in *PDX1* result in methylene blue sensitivity. Methylene blue, like cercosporin, is a photosensitizer, mediating the production of deleterious singlet oxygen molecules in light (Osmani et al., 1999). It was later shown, by animal blood assays, that vitamin B6 had three times the antioxidant activity of vitamin C and was shown to quench superoxide production (Jain and Lim, 2001; Stocker et al., 2003; Kannan and Jain, 2004). In rabbit lens cells, vitamin B6 prevented protein oxidation, a cause of cataract formation (Jain et al., 2002). As mentioned in section 1.1, vitamin B6 deficient rats exhibit elevated inflammatory responses due to acrodynia which also caused lipid peroxidation (Lakshmi et al., 1991).

**Vitamin B6 biosynthesis in plants**

Even though plants are known to be the major source of the vitamin in the human diet, information on its biosynthesis has been very limited. There was an early report claiming that deoxyxylulose (DX) is used for vitamin B6 formation in spinach chloroplasts (Julliard and Douce, 1991). But no proof of this reaction was provided. On the other hand, Leistner’s group (Drewke and Leistner, 2001), could not observe incorporation of isotopically labeled DX or DXP into the vitamin B6 derivative, 4-O-methylpyridoxine, in *Ginkgo biloba*. The identification of a pyridoxal kinase (PdxK) gene in *A. thaliana*, indicated that the salvage pathway is operational in plants (Shi et al., 2002). But none of the genes encoding *de novo* enzymes of vitamin B6 biosynthesis had been defined in plants.
Aim of the thesis and research strategy

From the complete genome sequence of *Arabidopsis thaliana*, our group has identified three putative *PDX1* family homologues; At2g38230, At3g16050, At5g01410 and one putative *PDX2* homologue At5g60540 which will be referred to as PDX1.1, PDX1.2, PDX1.3 and PDX2, respectively, for the remainder of this thesis.

Due to the shortage of information on vitamin B6 biosynthesis in plants, the aim of this research project was to study the biosynthesis of vitamin B6 in the model plant organism, *A. thaliana*. In particular, the functionality of the *PDX1* genes in vitamin B6 biosynthesis and whether they are essential for plant viability was to be investigated. As a means to reach this goal, a physiological and biochemical approach was chosen.
References


2 Vitamin B6 biosynthesis in higher plants


*These authors contributed equally to this work

Abstract

Vitamin B6 is an essential metabolite in all organisms. It can act as a coenzyme for numerous metabolic enzymes and has recently been shown to be a potent antioxidant. Plants and microorganisms have a de novo biosynthetic pathway for vitamin B6, but animals must obtain it from dietary sources. In Escherichia coli, it is known that the vitamin is derived from deoxyxylulose 5-phosphate (an intermediate in the nonmevalonate pathway of isoprenoid biosynthesis) and 4-phosphohydroxy-L-threonine. It has been assumed that vitamin B6 is synthesized in the same way in plants, but this hypothesis has never been experimentally proven. Here, we show that, in plants, synthesis of the vitamin takes an entirely different route, which does not involve deoxyxylulose 5-phosphate but instead utilizes intermediates from the pentose phosphate pathway, i.e., ribose 5-phosphate or ribulose 5-phosphate, and from glycolysis, i.e., dihydroxyacetone phosphate or glyceraldehyde 3-phosphate. The revelation is based on the recent discovery that, in bacteria and fungi, a novel pathway is in place that involves two genes (PDX1 and PDX2), neither of which is homologous to any of those involved in the previously doctrined E. coli pathway. We demonstrate that Arabidopsis thaliana has two functional homologs of PDX1 and a single homolog of PDX2. Furthermore, and contrary to what was inferred previously, we show that the pathway appears to be cytosolic and is not localized to the plastid. Last, we report that the single PDX2 homolog is essential for plant viability.
2.1 Introduction

Vitamin B6 is well renowned in the medical field for being involved in more bodily functions than any other single nutrient. The vitamin exists in various forms, i.e., pyridoxal, pyridoxine, pyridoxamine, and their phosphorylated derivatives. As pyridoxal 5′-phosphate, it is an essential cofactor for numerous metabolic enzymes including amino acid metabolism and antibiotic biosynthesis. Most interestingly, it has recently been found that the vitamin is a potent antioxidant with a particular ability to quench reactive oxygen species such as superoxide and singlet oxygen (1, 2). The de novo biosynthesis of vitamin B6 takes place in microorganisms and plants, but this ability has been lost in animals, making it essential in the human diet.

Despite the vitamin’s obvious physiological and pharmaceutical importance, its biosynthesis has predominantly been studied in the Gram-negative bacterium, *Escherichia coli*, where it is derived from deoxyxylulose 5-phosphate (DXP, an intermediate in the nonmevalonate pathway of isoprenoid biosynthesis) and 4-phosphohydroxy-L-threonine (refs. 3–7 and Scheme 1). Astonishingly, although plants are a major source of vitamin B6 in the human diet, our understanding of the pathway therein is very limited and has been assumed to be derived the same way as in *E. coli*. There is one preliminary study that reports on its biosynthesis in spinach (8), whereas other, more in-depth studies have addressed the topic based on the formation of 4′-O-methylpyridoxine, a vitamin B6 derivative found in *Ginkgo biloba* (9, 10). Knowledge of the pathway in plants may not only aid in studies designed for overproduction of the vitamin for beneficial effects, but proteins involved could also provide novel herbicidal targets if proven to be essential for viability of the organism.

Recently, it has become clear from genetic and labeling studies in fungi (2, 11–14) that a pathway alternative to the one in *E. coli* that does not involve DXP exists for vitamin B6 biosynthesis. Only two genes (*PDX1* and *PDX2*), which show no homology to any of the *E. coli* genes, appear to be involved in this alternative pathway (2, 15). Furthermore, based on extensive genomic analyses, it has been shown that the pathways of vitamin B6 biosynthesis are autoexclusive in that organisms have the genes for one or the other pathway but not both (2, 15). *PDX1* and *PDX2* have been predicted to function as a glutamine amidotransferase with *PDX2* as the glutaminase domain and *PDX1* as the acceptor/synthase domain. Indeed, glutaminase activity has been demonstrated for *PDX2* from a number of organisms (16–18). More recently, Burns et al. (19) and our own independent studies (51) have been able to reconstitute vitamin B6 formation from intermediates of glycolysis and the pentose phosphate pathway by using the *PDX1* and *PDX2* homologs from the Gram-positive bacterium *Bacillus subtilis*. 
Here, we address how vitamin B6 is synthesized in plants by using the model organism *Arabidopsis thaliana*. We report on the identification of homologs of *PDX1* and *PDX2* and demonstrate that, contrary to what is often quoted in the plant literature (e.g., refs. 20–22), vitamin B6 biosynthesis does not depend on the isoprenoid precursor DXP. Moreover, we demonstrate biosynthesis of the vitamin in a reconstituted system from *A. thaliana*. Last, we show that disruption of the single *PDX2* homolog is lethal for the plant resulting in arrested embryo development.

### 2.2 Materials and Methods

#### 2.2.1 Plant material

When grown in Petri dishes, *A. thaliana* seeds were surface sterilized and grown on Murashige and Skoog (MS) medium (23) (containing 1% wt/vol sucrose), either with or without vitamin B6 depending on the experiment, and under constant illumination at 22°C. WT (Col-2 or Ws), heterozygous SALK_072168, heterozygous RIKEN GSC 53-2381-1, and heterozygous *cla1-1* plants were maintained on soil under 16-h light/8-h dark cycles and genotyped by PCR. Homozygous *cla1-1* plants were obtained by allowing seeds from heterozygous plants to germinate in Petri dishes and selecting albino plants for analysis.

#### 2.2.2 Amplification of the genes

In the case of *PDX1.1-1.3*, the absence of introns allowed the direct amplification from genomic DNA isolated from *A. thaliana* ecotype Columbia, whereas *PDX2* was amplified from the isolated cDNA.

#### 2.2.3 Yeast complementation

The yeast strains used in this study were *Saccharomyces cerevisiae* BQS1037 and MML21, which are knockouts of *ScSNZ1* and *ScSNO1*, respectively, and were kindly provided by Enrique Herrero (Universitat de Valencia, Burjassot, Spain; ref. 24). For the complementation studies, the *AtPDX1.1-1.3* genes were cloned into the *PstI* and *NotI* sites of pCM262 (24), whereas *AtPDX2* was cloned into the *NotI* site of the same plasmid. Complementation was carried out on synthetic complete medium without vitamin B6 and with the appropriate selection markers. All plasmid constructs were maintained episomally.
**Scheme1.** Model depicting the DXP-dependent and –independent pathways of vitamin B6 biosynthesis

### 2.2.4 Transient expression and subcellular localization

Transient expression of GFP fusion proteins in onion epidermal cells was done according to the method of Scott et al. (25). Expression constructs were delivered into the cells by gold particle-mediated gun bombardment. Analysis of the expression in *Arabidopsis* protoplasts was performed according to ref. 26 by using 3- to 4-week-old plants maintained on MS plates but with some modifications: Incubation in enzyme solution was performed overnight, in the dark, and without agitation. Cells were allowed to recover 24–48 h before analysis by confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany) by using an ArKr laser at 488 nm. GFP fluorescence was recorded between 503 and 550 nm.

### 2.2.5 Analysis of the dependence of vitamin B6 biosynthesis in plants on DXP

Fosmidomycin (Molecular Probes) and clomazone (Riedel-de Haën, Seelze, Germany) inhibitor treatments were performed by growing seedlings for 7 days after imbibition on MS medium and then transferring them either to fresh MS medium (controls) or to MS medium containing the respective inhibitor. Vitamin B6 was always omitted from the MS medium. Seedling samples were harvested 96 h after inhibitor addition, frozen in liquid nitrogen, and stored at -80°C until further analysis. The amount of vitamin B6 in these samples and in wt and *cla1-1* plants was determined by using the microbiological assay as described in ref. 27, employing *S. carlsbergensis* American Type Culture Collection 9080. The vitamin was extracted from whole plant material (2 mg) by using 0.02 M H₂SO₄ as described in ref. 28 with the following modifications: After extraction the solution was neutralized to pH 5.2 and centrifuged,
and the supernatant was analyzed. Tissue from at least three independent experiments was used.

2.2.6 Recombinant expression, purification, and biochemical characterization of *A. thaliana PDX1.1–1.3*

The *PDX* genes were cloned into the *Ndel/XhoI* sites of pET21a (Novagen) in such a way as to allow expression of the proteins with a C-terminal hexa-histidine affinity tag in *E. coli* BL21 (DE3) cells. Expression was induced by addition of 0.1 mM isopropyl-1-β-thio-D-galactopyranoside, followed by growth for 5 h at 30°C. The proteins were purified by Ni-NTA chromatography (Qiagen) by using the nondenaturing protocol described by the manufacturer. The proteins were judged to be >90% homogeneous from an SDS/PAGE analysis. Enzyme assays were carried out in 50 mM Tris-HCl, pH 8, at 37°C containing 40 µM of the isolated protein and 500 µM of the respective substrates, ribose 5-phosphate or ribulose 5-phosphate, and either dihydroxyacetone phosphate or DL-glyceraldehyde 3-phosphate (1 mM) in the presence of 10 mM ammonium sulfate. The progress of the reaction was monitored from the appearance of the absorbance maximum at 414 nm. For the HPLC analysis (System Gold, Beckman), the reaction product was deproteinized by ultrafiltration, followed by precipitation with methanol-chloroform and applied to a C18 reversed phase column (LiChroCART 250-4, RP-18, 5 mM; Merck) in 0.1% trifluoroacetic acid. An isocratic elution, followed by a gradient of 0–50% acetonitrile, was used in all experiments. For the electron impact-MS, the reaction product was treated with alkaline phosphatase, deproteinized, purified by HPLC, and lyophilized. The spectrum was acquired on a Micromass Autospec Ultima instrument by using a source temperature of 200°C and with electrons accelerated at 70 eV.

2.2.7 Analysis of T-DNA insertion knockouts of *AtPDX2*

The *pdx2.1* mutant line was identified from the Salk Institute Genomic Analysis Laboratory T-DNA insertion lines (http://signal.salk.edu), and *pdx2.2* was identified from the available transposon tagged lines at the RIKEN BioResource Center (http://www.brc.riken.jp/lab/epd/Eng/catalog/seed.shtml). For the histological analysis, siliques were fixed with ethanol and acetic acid (3:1 vol/vol) overnight, followed by rehydration with ethanol and water (3:1 vol/vol). The defect was observed by clearing the siliques in chloral hydrate, glycerol, and water (10:1:2.5, wt/wt/wt) overnight and visualized with a compound microscope equipped with Nomarski optics.
2.3 Results and Discussion

2.3.1 Identification and functional characterization of the *Arabidopsis PDX1* and *PDX2* genes

Using BLAST with the *C. nicotianae PDX1* and *PDX2* sequences as queries (2, 11), three genes encoding putative PDX1s (At2g38230, At3g16050, and At5g01410) and a single gene encoding a putative PDX2 (At5g60540) were identified in the completely sequenced genome of *Arabidopsis*. For ease of reference, we propose to name the PDX1 homologs AtPDX1.1, AtPDX1.2 and AtPDX1.3, respectively, whereas the homolog of PDX2 will be referred to as AtPDX2. An alignment of the hypothetical *A. thaliana* PDX1 protein sequences (AtPDX1.1 shows 58% and 87% identity to AtPDX1.2 and AtPDX1.3, respectively) together with those from the fungi *C. nicotianae* (69%, 44%, and 68% identity with AtPDX1.1–1.3, respectively) and *S. cerevisiae* (58%, 41%, and 58% identity with AtPDX1.1–1.3, respectively) shows an extremely high degree of conserved sequence throughout the entire length of the genes (Figure 1A). Whereas AtPDX1.2 is not as strongly conserved as those of the other *A. thaliana* homologs, it also does not have the complete UPF0019 sequence LPVNFAGGAGAFFAPAAL, a characteristic of this protein family (www.ebi.ac.uk). An amino acid sequence alignment of PDX2 also shows a high degree of sequence identity between the plant and fungal homologs (35% and 25% identity between *Cercospora nicotianae* and *S. cerevisiae*, respectively) with almost the entire signature sequence of this protein family (UPF0030, [GA][L][LIV]PGGEST[STA]) being retained (Figure 1B). Importantly, the characteristic catalytic triad of class I glutamine amidotransferases, which consists of a cysteine, histidine, and glutamate residue in the glutaminase domain, is conserved in PDX2 (Figure 1B and refs. 29 and 30).

Expressed sequence tags can be found for all of these genes in a variety of tissues (http://mips.gsf.de). Moreover, total RNA isolation and real-time RT-PCR by using specific primers confirmed that all genes are expressed (Figure 6, which is shown as supporting information). To determine whether AtPDX1.1-1.3 and AtPDX2 encode functional enzymes, the genes were cloned into pCM262, a yeast expression vector under the control of a tetracycline regulatable promoter system (24, 31), and were tested for their ability to complement the *SNZ1* (functional PDX1 homolog) and *SNO1* (functional PDX2 homolog) disruption mutants, BQS1037 and MML21, respectively, from *S. cerevisiae* (24). When grown in minimal medium in the absence of vitamin B6, these mutants have a growth defect that is most pronounced in BQS1037 (24). Complementation of BQS1037 and MML21 with the *S. cerevisiae* *SNZ1* and *SNO1* genes, respectively, served as positive controls.
In the absence of vitamin B6, the snz1 mutant transformed with either *Arabidopsis* PDX1.1 or PDX1.3 grew significantly faster than the strain transformed with the empty vector (Figure 2A), suggesting that these plant homologs can restore vitamin B6 prototrophy in yeast. On the other hand, AtPDX1.2 did not restore prototrophy (Figure 2A). The sno1 mutant transformed with AtPDX2 showed enhanced growth in comparison to that of cells carrying the empty vector, indicating functional homology of the two genes (Figure 2B; growth in liquid culture is presented as Figure 7, which is shown as supporting information). When pyridoxine was included in the medium, all strains grew equally well (Figure 2).

Figure 1. The PDX1 and PDX2 proteins in *Arabidopsis*. Shown is the amino acid sequence alignment of the PDX1 (A) and PDX2 (B) homologs identified from *Arabidopsis*. Amino acids identical in at least two of the sequences are shaded in green (PDX1) and red (PDX2). The signature motifs of the respective protein class are underlined. The asterisk denotes the conservation of the Cys-His-Glu catalytic triad in PDX2. At, *A. thaliana*; Cn, *C. nicotianae*; Sc, *S. cerevisiae*

2.3.2 Subcellular localization of *AtPDX1.1–1.3* and *AtPDX2*

No clear targeting sequence could be identified in *AtPDX1.1–1.3* or *AtPDX2* from an *in silico* analysis. We therefore investigated the subcellular location of each of the proteins *in vivo* by fusing each one to the N terminus of GFP employing the vector pCAMBIA1302 (ref. 32; www.cambia.org) such that the fusions were under control of the CaMV35S promoter. We noted an extension at the C terminus of AtPDX2 when compared with homologous sequences of nonplant origin and, therefore, also investigated a C-terminal GFP fusion construct by using the
vector pSH11 (33). Each construct was introduced into onion epidermal cells and protoplasts of Arabidopsis mesophyll cells by particle gun bombardment and polyethylene glycol-mediated chemical transformation, respectively. The subcellular location of the transiently expressed GFP fusion proteins was viewed by confocal microscopy. As expected in the case of GFP alone, a diffuse fluorescence pattern typical of a cytosolic location was found in both the onion epidermal cells and Arabidopsis protoplasts with a tendency to accumulate in the nucleoplasm (Figure 3).

All three PDX1-GFP fusions and both GFP-PDX2 and PDX2-GFP exhibited a similar localization pattern (Figure 3), implying that they are cytosolic. It is clear from the Arabidopsis protoplast green fluorescence that there is no overlap with the chlorophyll autofluorescence nor did we observe a punctuate pattern typical of mitochondrial or microbody targeting in either the nonphotosynthetic (onion) or photosynthetic (Arabidopsis) tissue types. Western blot analyses of the transfected protoplasts by using a GFP antibody revealed that all of the fusion proteins remained intact (Figure 3).

**Figure 2.** The three *A. thaliana* PDX1 homologs identified and the single PDX2 homolog were tested for their ability to restore prototrophy to the *S. cerevisiae* strains BQS1037 and MML21. These strains are deletion knockouts in *SNZ1* and *SNO1*, which are functional PDX1 and PDX2 homologs in *S. cerevisiae*, respectively. (Left) Growth of 6-day-old BQS1037 (A) and 4-day-old MML21 (B) in the absence of vitamin B6. (Right) Growth of 3-day-old BQS1037 (A) and MML21 (B) in the presence of vitamin B6. Each row represents a serial 10-fold dilution from a starter culture with an OD at 600 nm of 0.5.

### 2.3.3 Vitamin B6 biosynthesis in plants occurs independent of deoxyxylulose 5-phosphate

It is generally assumed in the literature that deoxyxylulose 5-phosphate and 4-phosphohydroxy-L-threonine are the precursors for the formation of vitamin B6 in plants (20–22). Although it is well established that DXP is a precursor for the biosynthesis of Isoprenoids through the non-mevalonate (MEP) pathway in prokaryotes (34, 35) and plants (36–40), its role
in vitamin B6 biosynthesis has merely been inferred from extrapolation of the pathway as it occurs in *E. coli* (4, 5). There is only a single, very preliminary report (8) in the literature that claims that DX was used to form vitamin B6 in spinach chloroplasts, but no proof of the nature of the reaction product was provided. Using a microbiological assay, we measured the vitamin B6 content in the *Arabidopsis* *cla*-1 mutant. *CLA-1* encodes deoxyxylulose 5-phosphate synthase (DXS) (41, 42). Although the vitamin content of the *cla*-1 mutant plants is somewhat reduced compared with that of wild-type plants (Table 1), it is clear that vitamin B6 biosynthesis can still occur in the absence of this protein. In addition, the percentage of *cla*-1 seeds that germinate in the absence of exogenous vitamin B6 is almost identical to that of the wild type (data not shown). However, two additional genes, closely related to *CLA-1*, exist in *Arabidopsis* (designated *DXS2* and *DXS3*), which may encode DXP synthase but have not yet been functionally analyzed. Thus, the plants were treated with clomazone (Pestanal), an agent that has been shown to specifically inhibit DXP synthase (43).

<table>
<thead>
<tr>
<th>GFP</th>
<th>Bright-field</th>
<th>Overlay</th>
<th>GFP</th>
<th>Chlorophyll</th>
<th>Overlay</th>
<th>anti-GFP</th>
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<td><img src="image35" alt="anti-GFP" /></td>
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**Figure 3. Subcellular localization of *AtPDX1.1–1.3* and *AtPDX2*.** (Left) Transient expression of the indicated GFP fusion proteins in onion epidermal cells. (Center) The transient expression observed in isolated *A. thaliana* protoplasts. (Scale bars: 10 µm, unless indicated otherwise.) (Right) Western blot of crude extracts is shown from isolated *A. thaliana* protoplasts expressing the indicated constructs after immunodecoration with anti-GFP, indicating that the fusion proteins were intact.
A slight decrease comparable with that in the *cla-1* mutant plants was observed in the vitamin B6 content (Table 1). The observed decrease in vitamin B6 could indeed indicate that there are two routes functioning in its biosynthesis in plants (i.e., DXP dependent and DXP independent). However, an analysis of the available genomes has shown that there are no genes homologous to either *PdxA* or *PdxJ*, which catalyze formation of the pyridoxine ring in *E. coli* from DXP and 4-phosphohydroxy-L-threonine, in plants (and indeed most organisms with the exception of the γ-division of proteobacteria; refs. 2 and 15), arguing against the utilization of DXP in vitamin B6 biosynthesis. This conclusion is corroborated by the observation of E. Leistner’s group, referred to in ref. 9, that isotopically labeled DX or DXP was not incorporated into the vitamin B6 derivative, 4’-O-methylpyridoxine, in *G. biloba*. An explanation for the observed decrease in vitamin B6 can thus be that inhibition of DXP synthase depletes tissue of a metabolite that may positively regulate a key protein participating in the DXP-independent route. On the other hand, blocking the second enzyme in the non-mevalonate isoprenoid pathway, i.e., DXP reductoisomerase, would be expected to result in a buildup of DXP and, hence, vitamin B6. When wild-type plants were treated with fosmidomycin, which specifically inhibits DXP reductoisomerase (44, 45), no significant effect on the vitamin B6 content was observed (Table 1). Functionality of both clomazone and fosmidomycin as inhibitors under the conditions used was indicated by the bleaching phenotype observed after 48 h (Figure 8, which is presented as supporting information). This data indicates that vitamin B6 biosynthesis occurs in plants independent of DXP.

**Table 1.** Vitamin B6 content of WT, *cla1-1*, and inhibitor-treated *A. thaliana* plants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin B6, µg/gram of fresh weight</th>
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<tr>
<td>WT*</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td><em>cla1-1</em></td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>WT†</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Clomazone</strong>‡‡</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Fosmidomycin</strong>§§</td>
<td>5.2 ± 0.1</td>
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* Wassilewskija ecotype; † Columbia ecotype; ‡ Treated with 10⁻⁴ M clomazone for 96 hours; § Treated with 4x10⁻⁴ M fosmidomycin for 96 hours.
2.3.4 A pathway of vitamin B6 biosynthesis in plants

In yeast, the non-mevalonate pathway of isoprenoid biosynthesis does not exist (46). Moreover, the extensive studies of Spenser and colleagues (13, 14) indicate that in yeast, vitamin B6 biosynthesis proceeds by a route distinct from that in *E. coli* in that a pentose, which is not DXP, and a triose are the sugar precursors for formation of the vitamin in this organism. In the bacterial model (*B. subtilis*), it has recently been established by Burns et al. (19), and indeed by our own independent studies (51), that YaaD (the PDX1 homolog) can accept either ribose 5-phosphate or ribulose 5-phosphate as the pentose sugar and either dihydroxyacetone phosphate or glyceraldehyde 3-phosphate as the triose sugar to form vitamin B6 in the presence of YaaE (the PDX2 homolog) and glutamine. Here, we demonstrate the reconstitution of vitamin B6 biosynthesis from a higher plant. Recombinant *At*PDX1.1 and *At*PDX1.3 are able to catalyze the formation of the vitamin from either one of the above-mentioned pentose or triose sugars in the presence of ammonium sulfate as the nitrogen source and without addition of *At*PDX2 (Figure 4 A and B). No reaction was observed with glutamine under these conditions. Although the rate observed is substantially higher with ribose 5-phosphate and glyceraldehyde 3-phosphate in the presence of either PDX1 protein than with the other sugar molecules (Figure 4 A and B), *At*PDX1.3 appears to have a greater ability to accept dihydroxyacetone phosphate than *At*PDX1.1. Thus far, we have not been successful in isolating a functional recombinant version of *At*PDX2 based on the measurement of its ability to hydrolyze glutamine (16), but once achieved, the influence of *At*PDX2 on *At*PDX1 activity will be examined. No activity was observed with *At*PDX1.2 under any of these conditions, indicating that it is not a functional homolog. UV-visible spectrophotometric analysis of the reaction product by treatment with sodium borohydride or sodium hydroxide, results in shifts in the absorption maxima (from 414 nm to 317 nm and 390 nm, respectively) that are characteristic of pyridoxal 5'-phosphate (PLP) (Figure 4C). The identity of the reaction product under all conditions was confirmed by a HPLC analysis with commercially available standards (PLP, pyridoxal, and pyridoxine) and always resulted in coelution with pyridoxal 5'-phosphate (Figure 4D). Furthermore, the reaction product was dephosphorylated and submitted to electron impact-mass spectrometry. Although the molecular ion observed agrees very well with the calculated mass of the compound (168.0582), fragment masses corresponding to the loss of H$_2$O (149.0469) and the consecutive loss of the aldehyde group (120.0459), characteristic of pyridoxal, can be clearly observed (Figure 4 E and F). Because the reaction product of the functional *At*PDX1 is PLP, it can be concluded that the alternative vitamin B6 pathway observed in bacteria is also in operation in plants.
Figure 4. Biochemistry of vitamin B6 biosynthesis. (A and B) Initial rates showing vitamin B6 formation in the presence of ribose 5-phosphate (0.5 mM) and DL-glyceraldehyde 3-phosphate (1 mM) (solid line); ribulose 5-phosphate (0.5 mM) and DL-glyceraldehyde 3-phosphate (1 mM) (short dashed line); ribose-5-phosphate (0.5 mM) and dihydroxyacetone phosphate (0.5 mM) (long dashed line); ribulose 5-phosphate (0.5 mM) and dihydroxyacetone phosphate (1 mM) (dotted and dashed line) as measured in 50mM Tris-HCl, pH8.0, containing 10mM ammonium sulfate at 37°C, in the presence of AtPDX1.1 (40 µM) (A) and AtPDX1.3 (40 µM) (B). (C) UV-visible spectrum of the reaction product in 50 mM Tris, pH 8.0 (solid line); in the presence of sodium hydroxide (long dashed line); and in the presence of sodium borohydride (short dashed line). (D) HPLC analysis of (i) available standards that elute in the order PLP, pyridoxal, and pyridoxine; (ii) the reaction product (synthesized from ribulose 5-phosphate and dihydroxyacetone phosphate); and (iii) coelution of PLP and the reaction product. (E and F) Electron impact-MS of commercial pyridoxal (E) and the dephosphorylated reaction product (F). (F Inset) Characteristic fragmentation ions of pyridoxal. Note the masses due to the internal standard perfluorotributyl-n-amine can also be observed, e.g., 130.9922.
2.3.5 *AtPDX2* is essential for plant viability

Because *AtPDX2* is a single copy gene, various collections of T-DNA and transposon insertion mutants available were searched for a disruption in At5g60540, two of which are described here. SALK_072168 is a T-DNA insertion line (47), whereas 53-2381-1 is a transposon-tagged line from RIKEN GSC envisaged to have one insertion in the gene (48, 49). In the case of SALK_072168, genomic DNA blots revealed a complex insertion of three T-DNA’s at the same locus (data not shown). PCR analysis and sequencing established that the disruption in both lines occurs in the first intron as indicated in Figure 5A. For convenience, we will name these insertion alleles *pdx2.1* (SALK_072168) and *pdx2.2* (53-2381-1), respectively. Based on a PCR analysis, no homozygous plants could be identified in either the *pdx2.1* or *pdx2.2* collection of seeds, suggesting that seed development is impaired. A comparison of wild-type siliques with those of heterozygous *PDX2.1/pdx2.1* and *PDX2.2/pdx2.2* revealed that the heterozygous plants consistently had a population of albino seeds (Figure 5B). A statistical analysis of the heterozygous *PDX2.1/pdx2.1* and *PDX2.2/pdx2.2* seeds revealed that ~25% (± 4.0 and 1.3, respectively) showed the albino phenotype, thereby representing the homozygous population. As the albino phenotype could be either due to the loss of pigment(s) or developmental arrest, a histological analysis was carried out. In cleared whole-mount specimens, *pdx2.1* and *pdx2.2* seeds were developmentally delayed compared with their wild-type or heterozygous siblings. The developmental arrest in all seeds analyzed occurred at the globular stage of embryo development (Figure 5C). Progress to the heart stage was never observed in *pdx2.1* or *pdx2.2* even though they were monitored from the globular to the cotyledon stage of embryo development. As far as could be judged, the heterozygous plants developed normally from seedling stage to maturity. The arrest in development observed in *pdx2.1* and *pdx2.2* can be explained by the assumption that during the early stages of embryogenesis the surrounding maternal tissues supply vitamin B6 among other nutrients, but that the globular to heart transition may be a critical stage in the conversion from heterotrophy to autotrophy. In this context, many of the metabolic enzymes likely to be required at this stage have pyridoxal 5’-phosphate as an essential cofactor, e.g., tryptophan synthase and ornithine decarboxylase involved in tryptophan and spermine and spermidine biosynthesis, respectively, to name but two. It is noted that an allele of *pdx2.1* and *pdx2.2* has also been described in the Seedgenes database (www.seedgenes.org) where it is reported that a T-DNA insertion in exon 2 of At5g60540 results in arrest of embryo development. We have now initialized experiments to rescue the homozygous phenotype by enriching complete MS growth medium with vitamin B6. However, it is notoriously difficult to rescue embryos arrested at such an early stage of
development and may rely on the generation of embryos that can at least reach the globular to heart stage transition (50).

Figure 5. Phenotype of the Atpdx2 knockout. (A) Exon-intron structure of PDX2 from A. thaliana indicating the location of the insertions of RIKEN GSC 53-2381-1 (▼) and SALK_072168 (▼). (B) Wild-type immature silique showing normal seeds (Upper), in comparison with one from a heterozygous plant PDX2.1/pdx2.1 (Lower), showing the albino phenotype (indicated arrowheads). (C) Cleared whole-mounted seeds from PDX2.1/pdx2.1 plants, WT or heterozygote at the heart (Upper) and torpedo (Lower) stages and pdx2.1 embryos arrested at the globular stage. The pictures shown are of pdx2.1 (SALK_072168), but analysis of pdx2.2 (RIKEN GSC 53-2381-1) indicated the same phenotype.

2.4 Concluding remarks

In this report, we show that, contrary to what is generally tacitly assumed, vitamin B6 biosynthesis in plants does not appear to depend on DXP. Instead, the vitamin is synthesized from the pentose phosphate pathway intermediates, ribose 5-phosphate or ribulose 5-phosphate, and either glyceraldehyde 3-phosphate or dihydroxyacetone phosphate (Scheme 1). Moreover, it appears that this pathway is cytosolic, rather than plastidial as was inferred in ref. 8. Of the genes that characterize this previously undescribed pathway, two functional homologs of
PDX1 and a single copy of PDX2 are found in Arabidopsis; the latter is essential for plant viability. To summarize, light has been shed onto how this essential vitamin can be synthesized in plants.

2.5 Acknowledgements

We dedicate this study to Ian Spenser in appreciation of his pioneering work on vitamin B6 biosynthesis. We thank Dr. Enrique Herrero for providing the yeast strains, the pCM262 plasmid and the positive controls used in the yeast complementation experiments; Dr. Cristophe Laloi for his general help and advice with Arabidopsis; Andreas Furholz and Oliver Laule (Swiss Federal Institute of Technology) for their kind gift of the cla1-1 seeds; the RIKEN BioResource center for supplying line 53-2381-1 for analysis and likewise the Salk Institute for providing line 072168; Philippe Roy for performing the HPLC analysis; Oswald Greter and Bernhard Stump for acquiring the Electron Impact-MS; and Dr. Joanna Wyrzykowska for help with the histological analysis. This work was supported by ETH Zurich Grant 0094/41h2703.5 and Swiss National Science Foundation Grant 3100A0-107975/1.

2.6 Supporting information

Additional experimental procedures used in this study are provided in more detail in the following supporting text. The primers used in this study, including the incorporated restriction sites if applicable, are listed below. PCR reactions were performed using either Vent polymerase (New England Biolabs) or Taq polymerase (Roche).

Cloning into pCM262 for complementation in yeast:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction site used</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDX1.1</td>
<td>NotI / 5’-3’</td>
<td>ATAAGAATGCGGCCGCGCATGGCAGGAACCGGAGTTGTGGC</td>
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<tr>
<td>PDX1.1</td>
<td>PstI / 5’-3’</td>
<td>AACTGCAGTTACTCAGAACGACTAGCGAACCTCTCAACCTTATC</td>
</tr>
<tr>
<td>PDX1.2</td>
<td>NotI / 5’-3’</td>
<td>ATAAGAATGCGGCCGCGCATGGCAGGATCAAGCTATGACGGATC</td>
</tr>
<tr>
<td>PDX1.2</td>
<td>PstI / 5’-3’</td>
<td>AACTGCAGTCAAACACTGCGTATGGCCAAAGTC</td>
</tr>
<tr>
<td>PDX1.3</td>
<td>NotI / 5’-3’</td>
<td>ATAAGAATGCGGCCGCGCATGGAAGGAACCGGAGTTTTGAGCGG</td>
</tr>
<tr>
<td>PDX1.3</td>
<td>PstI / 5’-3’</td>
<td>AACTGCAGTCAAACACTGCGTATGGCCAAAGTC</td>
</tr>
<tr>
<td>PDX2</td>
<td>NotI / 5’-3’</td>
<td>ATAAGAATGCGGCCGCGCATGGAAGGAACCGGAGTTGTGAGTCTTTG</td>
</tr>
<tr>
<td>PDX2</td>
<td>NotI / 5’-3’</td>
<td>ATAAGAATGCGGCCGCGCATGGAAGGAACCGGAGTTGTGAGTCTTTG</td>
</tr>
</tbody>
</table>

It should be noted that in each case, transformants were grown on medium lacking vitamin B6 for three generations to deplete the cells of any residual vitamin before complementation analysis.
Transient expression of GFP-fusion proteins

For cloning into pCAMBIA1302 to create C-terminal fusions to GFP the primers used were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction site used</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDX1.1</td>
<td>NcoI / 5'-3'</td>
<td>CATGCCATGGCAGGAACCGGAGTTGTGGCGGTGTACGGGCG</td>
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<tr>
<td>PDX1.1</td>
<td>SpeI / 5'-3'</td>
<td>GGACTAGTCTCAGAAGACTAGCGAACCTCTTC</td>
</tr>
<tr>
<td>PDX1.2</td>
<td>NcoI / 5'-3'</td>
<td>CATGCCATGGCGATCAAGCTATGACGGGATC</td>
</tr>
<tr>
<td>PDX1.2</td>
<td>SpeI / 5'-3'</td>
<td>GGACTAGTAACACTGCCCTTGCGCAAGTTCTTC</td>
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<tr>
<td>PDX1.3</td>
<td>NcoI / 5'-3'</td>
<td>CATGCCATGGAAAGGAACCGCGGTGGCGGTGTACGGTAACGG</td>
</tr>
<tr>
<td>PDX1.3</td>
<td>SpeI / 5'-3'</td>
<td>GGACTAGTTCTCAGAAGGAGTTCTTATCTCTT</td>
</tr>
<tr>
<td>PDX2</td>
<td>NcoI / 5'-3'</td>
<td>GAAGATCTATAGCCGTCGGATTTAGGTTCTT</td>
</tr>
<tr>
<td>PDX2</td>
<td>SpeI / 5'-3'</td>
<td>GGACTAGTTTGAATATAGGGAAGATCACAGGCTTC</td>
</tr>
</tbody>
</table>

The AtPDX2 gene was also cloned into pSH11, a derivative of pBluescript SK+ (Stratagene) containing as expression cassette consisting of the 35S promoter and the NOS terminator for expression for EGFP (Clontech), which was kindly provided by Prof. A. Schaller (Universität Hohenheim, Germany). In this case AtPDX2 was amplified by PCR using the synthetic oligonucleotides 5'-ATAAGAATGCGGCCGCAACCGTCGGAGTTTATAGGCTTTG-3' and 5'-ATAAGAATGCGGCCGCTTATTAGGAAATATTAGG-AAGATCACG-3' and cloned downstream of EGFP ORF using a NotI restriction site. The joined ORFs encode for a fusion protein containing EGFP and PDX2 (without its first Met) at its N- and C- termini, respectively.

Heterologous expression of the recombinant AtPDX1.1-1.3 in E. coli

Primers used were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction site used</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDX1.1</td>
<td>NdeI / 5'-3'</td>
<td>GGAATTCCATATGCGAGAAGCTGCAGTGATTTGCGGCG</td>
</tr>
<tr>
<td>PDX1.1</td>
<td>XhoI / 3'-5'</td>
<td>CCGCTCGAGACTCGAGAAGCTGCAGTGATTTGCGGCG</td>
</tr>
<tr>
<td>PDX1.2</td>
<td>NdeI / 5'-3'</td>
<td>GGAATTCCATATGCGAGAAGCTGCAGTGATTTGCGGCG</td>
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<td>CCGCTCGAGACTCGAGAAGCTGCAGTGATTTGCGGCG</td>
</tr>
<tr>
<td>PDX1.3</td>
<td>NdeI / 5'-3'</td>
<td>GGAATTCCATATGCGAGAAGCTGCAGTGATTTGCGGCG</td>
</tr>
<tr>
<td>PDX1.3</td>
<td>XhoI / 3'-5'</td>
<td>CCGCTCGAGACTCGAGAAGCTGCAGTGATTTGCGGCG</td>
</tr>
</tbody>
</table>

Genetic analysis of Arabidopsis plants

DNA extraction and PCR analysis was performed on two-week-old plant leaf material. The offspring of segregating plants PDX2.1/pdx2.1 were genotyped using the following primers: 5'-ATGACCCTGGACTGGTCGTTTAGGTCGTTTAC-3' and 5'-GACTTCGACATCAGG-ACCTACATC-3' for the
wild type fragment; 5'-GCGTGGACCGCTTGCTGCAACT-3' (L Bb1, Salk) and 5’-
GACTTCGACATCAGGACCTACATC-3’ for identifying the presence of the insertion. For
Southern blots, 5 µg of genomic DNA extracted from heterozygote plants was digested overnight
with 80 U of EcoRI and HindIII, followed by electrophoretic separation and then blotted onto
nitrocellulose (Hybond N+, Amersham). The blots were probed separately with fragments
specific to the T-DNA insertion and to the PDX2 gene. Wild type plants were used as a negative
control in every experiment. The probes were DIG-labeled using the PCR DIG Probe Synthesis
Kit (Roche).

Figure 6. Expression data of AtPDX1.1, AtPDX1.2, AtPDX1.3 and AtPDX2 in rosette leaves
using real time RT-PCR. Total RNA was extracted from rosette leaves using the RNeasy mini kit
(Qiagen). Traces of DNA were removed using a RNase-Free DNase Set Kit (Qiagen). The
amount of total RNA was measured spectrophotometrically. The first strand cDNA synthesis was
obtained by using 1 µg of total RNA and the oligo(dT) and reverse transcriptase provided in the
Advantage RT-for-PCR kit (Clontech). The transcripts were quantified using fluorescence-based
real-time PCR using the fluorescent dye SYBR-green. To correct for sample variations, the
relative gene expression levels were normalized against the expression of the constitutively
expressed gene actin (At3g18780). Specific primers used for this quantification were: AtPDX1.1
forward, 5’ GTGAGGAGTGTGAACGGAGC 3’; AtPDX1.1 reverse, 5’
GCACAACCAATCATACGGC 3’; AtPDX1.2 forward, 5’ GATGCAGCTAGGTTGTGATGG 3’;
AtPDX1.2 reverse, 5’ TCCATTGCATTCTCCAATCC 3’; AtPDX1.3 forward, 5’
ATAATTTCCGGATCCCGTTC 3’; AtPDX1.3 reverse, 5’ CATCATCATCCATGTTTCGC 3’;
AtPDX2 forward, 5’ AGATGGGGAAACCTGTTTGG 3’; AtPDX2 reverse, 5’
CTGTATGTCTCTGGCCCACC 3’; Actin forward, 5’ ATTCTTGCTTCCCTCAGCACC 3’; Actin
reverse, 5’ CCCAGCCTTTTTAAGCCTTT 3’.
Figure 7. Growth of MML21 (24), a knockout strain of ScSNO1, in medium lacking vitamin B6. For the complementation studies MML21 was transformed with pCM262-AtPDX2(•), pCM262-ScSNO1 (x) and the empty vector pCM262 (•) as a negative control. The transformants were grown in liquid medium at 30°C from a starter culture at an O.D. at 600 of 0.4. The optical density was measured at the indicated time intervals.

Figure 8. Effect of inhibitor treatments on A. thaliana seedlings. WT seeds (ecotype Columbia) were surface sterilized and grown on Murashige and Skoog (MS) medium containing 1% sucrose for 7 days after imbibition. The seedlings were then transferred to fresh MS medium (control) or fresh MS medium containing 100 μM clomazone (43) or 400μM fosmidomycin (21). In all cases vitamin B6 was omitted from the medium. The pictures shown are 48 hours after treatment and demonstrate an effective concentration based on the bleaching phenotype observed.
2.7 References

3 PDX1 is essential for vitamin B6 biosynthesis, development and stress tolerance in *Arabidopsis*


*These authors contributed equally to this work

Abstract

Vitamin B6 is an essential coenzyme for numerous metabolic enzymes and is a potent antioxidant. In plants, very little is known about its contribution to viability, growth and development. The *de novo* pathway of vitamin B6 biosynthesis has only been described recently and involves the protein PDX1. *Arabidopsis thaliana* has three homologs of PDX1, two of which, PDX1.1 and PDX1.3, have been demonstrated as functional in vitamin B6 biosynthesis *in vitro* and by yeast complementation. In this study, we show that the spatial and temporal expression patterns of *PDX1.1* and *PDX1.3*, investigated at the transcript and protein level, largely overlap, but PDX1.3 is more abundant than PDX1.1. Development of single *pdx1.1* and *pdx1.3* mutants is partially affected, whereas disruption of both genes causes embryo lethality at the globular stage. Detailed examination of the single mutants, in addition to those that only have a single functional copy of either gene, indicate that although these genes are partially redundant in vitamin B6 synthesis, *PDX1.3* is more requisite than *PDX1.1*. Developmental distinctions correlate with the vitamin B6 content. Furthermore, we provide evidence that in addition to being essential for plant growth and development, vitamin B6 also plays a role in stress tolerance and photoprotection of plants.

3.1 Introduction

The term vitamin B6 collectively refers to the vitamers pyridoxal, pyridoxine, pyridoxamine, and their respective phosphate esters (Rabinowitz and Snell, 1941). The vitamin is renowned in
the medical field as being involved in more bodily functions than any other single nutrient being required for the maintenance of physical as well as mental health (Gengenbacher et al., 2006). The phosphorylated forms pyridoxal 5´-phosphate (PLP) and pyridoxamine 5´-phosphate (PMP) are active as enzyme cofactors. PLP, in particular, is well established as a cofactor for enzymes involved in amino acid, lipid and carbohydrate metabolism (John, 1995).

Recently, the vitamers have been shown to be potent antioxidants equivalent to vitamins C and E and are particularly active in quenching singlet oxygen and the superoxide anion (Ehrenshaft et al., 1999; Osmani et al., 1999; Jain and Lim, 2001). The de novo synthesis of the vitamin is now known to occur through either of two pathways (Lam and Winkler, 1990, 1992; Zhao and Winkler, 1996; Cane et al., 1998; Cane et al., 1999; Laber et al., 1999; Burns et al., 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005), which can be referred to as DXP-dependent and DXP-independent (Tambasco-Studart et al., 2005). The DXP-dependent pathway is found in Escherichia coli and many other members of a small subset of the γ division of proteobacteria (Ehrenshaft et al., 1999; Mittenhuber, 2001) and has been studied for decades. This pathway involves six steps, catalyzed by GapA, PdxB, PdxF, Dxs, PdxA and PdxJ, which result in the formation of pyridoxine 5´-phosphate (PNP) from intermediates of glucose metabolism (for reviews see (Drewke and Leistner, 2001; Mittenhuber, 2001)). The DXP-independent pathway, on the other hand has been only recently discovered and appears to be the predominant route being found in archaea, most bacteria, fungi and plants (Ehrenshaft et al., 1999; Osmani et al., 1999; Mittenhuber, 2001). It involves only two enzymes, i.e. PDX1 and PDX2, which function as a glutamine amidotransferase resulting in the direct formation of PLP from intermediates of glycolysis and the pentose phosphate pathway (Burns et al., 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005). Interestingly, it appears that either one or the other pathway is present in any one organism but not both (Ehrenshaft et al., 1999; Mittenhuber, 2001).

By definition, animals must take the vitamin up in their diet. The different vitamers can be inter-converted via the so-called salvage pathway (Hill and Spenser, 1996; Yang et al., 1996; Yang et al., 1998). This pathway appears to exist in most organisms and comprises PdxH, an oxidase which is active on PMP and PNP converting them to PLP (Zhao and Winkler, 1995); PdxK, a kinase that catalyses the conversion of pyridoxine (PN), pyridoxal (PL) or pyridoxamine (PM) to the respective 5´-phosphate ester (Yang et al., 1996), and an apparently specific PL kinase, PdxY (Yang et al., 1998). Somewhat surprisingly, plants defective in vitamin B6 biosynthesis have been described only recently (Shi et al., 2002; Shi and Zhu, 2002; Chen and Xiong, 2005; Tambasco-Studart et al., 2005). The first to be reported was that of the salt-sensitive mutant sos4, which carries a mutation in PdxK resulting in impaired root hair development and increased sensitivity to Na+, K+ and Li+ ions. However, despite much research
(Fiehe et al., 2000; Drewke and Leistner, 2001), very little information was available on the \textit{de novo} pathway of vitamin B6 biosynthesis in plants until recently (Tambasco-Studart et al., 2005). It was only with the discovery of the DXP-independent pathway (Ehrenshaft et al., 1999; Osmani et al., 1999; Ehrenshaft and Daub, 2001; Burns et al., 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005) and the characterization of this pathway in \textit{Arabidopsis} that we could show that knocking out the single \textit{PDX2} homolog is lethal for the plant resulting in the arrest of embryo development at the globular stage (Tambasco-Studart et al., 2005). More recently, a \textit{pdx1} mutant was shown to be affected in root and leaf development and to have an increased sensitivity to salt, osmotic and oxidative stress (Chen and Xiong, 2005). However, there are three homologs of \textit{PDX1} in \textit{Arabidopsis} (\textit{PDX1.1}, \textit{PDX1.2}, \textit{PDX1.3}). Two of these (\textit{PDX1.1}, \textit{PDX1.3}) have been shown to be functional in vitamin B6 biosynthesis (Tambasco-Studart et al., 2005).

Here we provide a more detailed analysis of the functional \textit{PDX1} genes in \textit{Arabidopsis} by comparing their expression patterns at both the RNA and protein level. We report on the characteristics of the individual and double knockouts of the genes. We show that \textit{PDX1.3} is necessary for post-embryonic root and shoot development and is also required for plant tolerance to abiotic stress, while loss of \textit{PDX1.1} does not have such drastic consequences. The absence of both proteins results in embryo lethality. Plants that have only a single copy of either gene show a severe delay in growth and development that is more pronounced in plants that do not carry a copy of \textit{PDX1.3}. Despite the growth retardation, plants that have a single copy of \textit{PDX1.3} perform the switch from vegetative to reproductive growth at approximately the same time as wild-type, whereas the switch in single copy \textit{PDX1.1} plants is impeded. Finally, we show that the particular role of \textit{PDX1.3} in stress tolerance appears to correlate with the vitamin B6 content of the plant, and we furthermore provide indirect evidence that vitamin B6 may play a role in the photoprotection of plants.

3.2 Results

3.2.1 Expression analysis reveals that \textit{PDX1.3} is more abundant than \textit{PDX1.1}

Expression of \textit{PDX1.1} (At2g38230), \textit{PDX1.2} (At3g16050) and \textit{PDX1.3} (At5g01410) was analyzed in various organs of the plant, i.e. roots, stem, cauline leaves, rosette leaves, flowers, siliques and cotyledons at the RNA level using quantitative RT-PCR, and at the protein level using specific antibodies. Both \textit{PDX1.1} and \textit{PDX1.3} transcripts could be detected in all organs
examined (Figure 1a, upper panel). Notably, the lowest level of both *PDX1.1* and *PDX1.3* transcript was detected in the root whereas highest levels were found in cotyledons and siliques, respectively. This is in direct contrast to the *PDX1.2* transcript, which is predominantly found in the root and shows a relatively low abundance in the above ground organs, being found mainly in flowers and siliques (Figure 1a, lower panel). The *PDX1.3* transcript appeared to be present at a generally higher level than that of *PDX1.1*, as confirmed by the relative abundances of EST’s found in databases, 103 and 37 respectively, as well as the averages of the signal intensity values from publicly available microarray data, 7761 and 3658 respectively (Zimmermann et al., 2004).

Expression of *PDX1.1* and *PDX1.3* was also assessed at the protein level using an antibody directed against the homologous *Bacillus subtilis* Pdx1 protein (64% identity), which had been affinity purified against both the PDX1.1 and PDX1.3 recombinant proteins. This antibody has similar affinity for all three PDX1 homologs, but they can be distinguished by their mobility during SDS-PAGE (data not shown). The proteins migrate with mobility from lowest to highest in the order PDX1.2<<PDX1.1<PDX1.3. The PDX1.2 protein is much less abundant than either PDX1.1 or PDX1.3, such that only the two latter proteins are observable in plant protein extracts under the conditions used. Both proteins could be detected in most of the above ground tissues examined, with PDX1.3 being more abundant in general than PDX1.1 (Figure 1a, centre panel). Immunodecoration of the proteins was barely detectable in the roots under the conditions used, most likely due to their lower abundance in this organ. In most cases it appears that there is a reasonable correlation between the level of protein and of transcript.
Figure 1. Expression analysis of PDX1. *Upper panel:* (a) Quantitative RT-PCR analysis of PDX1.1 or PDX1.3 transcript abundance in roots, stems, cauline leaves, rosette leaves, flowers, siliques and cotyledons. The values indicated are the average mRNA level relative to that of PDX1.1 in roots (value=1). (b) Time course of expression in rosette leaves of two, four, six, eight and eleven-week old plants, respectively. The values indicated are the average mRNA level relative to that of PDX1.1 at two-weeks (value=1). (c) Expression in etiolated seedlings exposed either to continuous light (LL) or continuous dark (DD), and 15 minutes, 1 hour, 5 hours, and 24 hours after a shift from dark to light, respectively. The values indicated are the average mRNA level relative to that of PDX1.1 in LL (value=1). In each case, total RNA was extracted either from (a) the tissue being analyzed or (b) rosette leaves or (c) 5-day old etiolated seedlings and probed with primers specific to PDX1.1 (black bars) or PDX1.3 (grey bars). Error bars indicate the standard deviation of the experiment performed in triplicate. *Centre panel:* Immunochemical analysis of PDX1.1 and PDX1.3 expression. Total protein was extracted from the same samples as in the upper panel and probed with an antibody raised against BsPdx1 that had been affinity purified before use against both PDX1.1 and PDX1.3. The three PDX1 paralogs can be distinguished by their mobility on SDS-PAGE (PDX1.3<PDX1.1<<PDX1.2). PDX1.2 is not visible under the conditions used due to its relatively low abundance compared to PDX1.1 and PDX1.3. Thirty µg of total protein was loaded per lane. *Lower panel:* Quantitative RT-PCR analysis of PDX1.2 transcript abundance. The samples are the same as in the upper panel but probed with primers specific to PDX1.2. The values indicated are the average mRNA level relative to that of (a) PDX1.2 in cotyledons (value=1); (b) PDX1.2 at two weeks (value=1); (c) PDX1.2 in continuous light (value=1).

We also analyzed expression of the genes in the rosette leaves of developing plants (Figure 1b). In this case, we noted that the correlation between transcript and protein of PDX1.1 and PDX1.3 is reduced, which may be indicative of post-transcriptional or post translational...
regulation during development. At the protein level, the expression appears to be strongest at younger stages, followed by a gradual decline until senescence (Figure 1b, centre panel). This pattern is most pronounced in the case of PDX1.3, as is its predominance compared to PDX1.1, throughout the life cycle. In contrast, the PDX1.2 transcript accumulates during senescence (Figure 1b, lower panel). We also observed that the level of PDX1.1 and PDX1.3 is dramatically reduced in etiolated seedlings compared to those grown under continuous light (Figure 1c, upper and centre panel). Upon illumination, expression of both PDX1.1 and PDX1.3 is induced in a time-dependent manner and can be observed at both the transcript and translational level. On the other hand, expression of PDX1.2 does not appear to be strongly induced by light (Figure 1c, lower panel). A survey of the expression of the PDX1 genes using the Arabidopsis microarray database Genevestigator (Zimmermann et al., 2004) confirmed our analysis, i.e. a good correlation between the expression pattern of PDX1.1 and PDX1.3, while PDX1.2 shows a pattern very different from that of either of the other genes (Figure S1). However, we noted that differences could also be observed in the expression between PDX1.1 and PDX1.3 (e.g. specific expression of PDX1.3 in pollen). We next decided to focus on a more detailed analysis of PDX1.1 and PDX1.3, in particular to examine the effect (if any) on plant viability in their absence.

3.2.2 Identification and isolation of pdx1.1 and pdx1.3 mutant plants

We have previously reported that knocking out the single PDX2 gene in Arabidopsis is lethal for the plant (Tambasco-Studart et al., 2005). To investigate if the absence of either PDX1.1 or PDX1.3 would have a similar effect on plant viability, we identified insertion mutants from the available libraries (Figure 2a). In the case of PDX1.1 we characterized both the SM_3_22664 line (pdx1.1-1) from the SLAT collection (Tissier et al., 1999) and line 423C02 (pdx1.1-2) from the GABI collection (Rosso et al., 2003), which are predicted to have a disruption within the gene coding sequence. The line SM_3_22664 was generated by independent insertions of the dSpm transposon and introduces a BAR gene conferring resistance to phosphinothricin (Basta™). On the other hand, GABI_423C02 is a T-DNA insertion line that carries the SUL1 gene conferring resistance to sulfadiazine. Polymerase chain reaction analysis with primers designed to hybridize close to either end of the inserted element and the flanking gene sequence revealed bands of the expected size thus confirming the respective insertion(s) in PDX1.1. From this line, plants homozygous for the insertion(s) could be identified (Figure 2b). Sequencing of the amplified fragments revealed that the Ds and T-DNA insertions are located at base pair 295 and 590 relative to the start codon, respectively. The SM_3_22664 line was investigated in more detail.
Figure 2. Identification and characterization of *pdx1.1* and *pdx1.3*. (a) Diagram of *PDX1.1* and *PDX1.3* indicating the position of the respective insertions relative to the start codon. (b) Plant genotypes. In each panel, lanes 1 and 3 show results of PCR reactions that were designed to detect the dSpm transposon insertion in *pdx1.1-1* (SM_3_22664), the T-DNA insertion in *pdx1.1-2* (GABI_423C02), or the T-DNA insertion in *pdx1.3* (SALK_086418), respectively, of the line in question versus wild-type. Lanes 2 and 4 show the results for the presence of a wild-type copy of either *PDX1.1* or *PDX1.3* in the respective lines versus wild-type. Only wild-type and plants homozygous for the respective *PDX1* insertions are shown. (c) Transcript abundance of *PDX1* in wild-type, *pdx1.1* or *pdx1.3* plants, respectively, as analyzed by quantitative RT-PCR. Total RNA was extracted from 14-day old seedlings grown in sterile culture and probed with primers specific to *PDX1.1* (black bars) or *PDX1.3* (grey bars). The values indicated are the average mRNA level relative to *PDX1.1* in wild-type (value=1). Error bars indicate the standard deviation of the experiment performed in triplicate. (d) Western blot analysis. Total protein was extracted from the same samples as in (c) and probed with an antibody raised against *Bacillus subtilis* Pdx1 that had been affinity purified before use against both *PDX1.1* and *PDX1.3*.

A Southern blot analysis revealed the presence of a single insertion (Figure S2). A segregation analysis among the progeny of selfed *PDX1.1/pdx1.1-1* plants revealed that Basta™-resistant and Basta™-sensitive seedlings segregated in a ratio of 3:1 (Table 1) corroborating that there is a single transposon in the single locus. In the case of *PDX1.3*, we characterized line 086418 from the SALK collection (Alonso et al., 2003), which has an insertion at base pair 160 in the coding sequence (Figure 2a). A molecular analysis of this line indicated that it has three T-DNAs.
inserted in tandem at the same locus (Figure S2). A quantitative RT-PCR analysis revealed that the respective transcript in the *pdx1.1* lines and the *pdx1.3* line was severely reduced (Figure 2c). Furthermore, the correspondingly ascribed protein band could not be detected in the respective insertion lines by Western blot analysis using the affinity purified Pdx1 antibody (Figure 2d).

**3.2.3 PDX1.1 and PDX1.3 are required for shoot and root development**

Plants homozygous for an insertion in *PDX1.1* or *PDX1.3* (Figure 2b) were assessed for a distinctive phenotype when grown on soil or in sterile culture. On soil, we observed that while the above ground organs of *pdx1.1-1* and *pdx1.1-2* appeared to develop similar to wild-type, the developing rosette leaves of *pdx1.3* were slightly chlorotic (Figure 3a and Figure 5a). However, this did not appear to affect further development (Figure 3b). Siliques of *pdx1.1-1* showed a full normal seed set. In contrast, siliques of *pdx1.3* had a significant increase in aborted seeds implying that fertility might be impaired (Table 2). When grown in sterile culture in the absence of vitamin B6, *pdx1.3* seedlings showed reduced shoot growth and had a distinct pale green phenotype, while *pdx1.1-1* and *pdx1.1-2* appeared normal (Figure 3c and Figure 4). In addition, under these conditions, *pdx1.3* is strongly reduced in primary root growth (Figure 3c), as has been described by Chen and Xiong (2005). Monitoring of *pdx1.3* for a longer period (up to 22 days in sterile culture) revealed that while overall development is severely delayed, the retarded growth appears to be more pronounced for the root rather than the shoot (Figure S3). Interestingly, while an impairment of root development could be observed both in *pdx1.1-1* and *pdx1.1-2*, it was not as explicit as with *pdx1.3* (Figure 3c). A comparison of the rates of root elongation in *pdx1.3*, *pdx1.1-1* and *pdx1.1-2*, with that of wild-type revealed a reduction of 92%, 27% and 19%, respectively (Figure S3). Furthermore, measurement of the vitamin B6 content indicated a higher reduction in *pdx1.3* in both soil and sterile culture in comparison to either *pdx1.1* or the wild-type (Figure 5b and c). Supplementation of the sterile culture with 5 µM pyridoxine restores growth of *pdx1.1-1*, *pdx1.1-2* and *pdx1.3* to that observed with the wild type under the same conditions (Figure 3c and Figure 4), confirming that the observed phenotype is due to a vitamin B6 deficiency.
Table 1 Segregation ratio of the BAR gene conferring phosphinothricin herbicide resistance in transposant SM_3 22656 (pdx1.1-1)

<table>
<thead>
<tr>
<th>Line</th>
<th>BASTA(^1)</th>
<th>BASTA(^2)</th>
<th>Total</th>
<th>(P) Value</th>
</tr>
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<tr>
<td>10</td>
<td>172</td>
<td>70</td>
<td>242</td>
<td>0.16</td>
</tr>
<tr>
<td>11</td>
<td>251</td>
<td>90</td>
<td>341</td>
<td>0.54</td>
</tr>
</tbody>
</table>

The progeny of selfed heterozygous line SM_3 22656 were analyzed for resistance and sensitivity to BASTA\(^TM\), the \(P\) value calculated is based on a 3:1 BASTA\(^1\) :BASTA\(^2\) segregation ratio.

Table 2 Analysis of the fertility of pdx1.1 and pdx1.3

<table>
<thead>
<tr>
<th></th>
<th>Normal seeds</th>
<th>Non-fertilized ovules</th>
<th>Seeds scored</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdx1.1-1</td>
<td>97.5%</td>
<td>2.5%</td>
<td>1975</td>
<td>0.12</td>
</tr>
<tr>
<td>pdx1.3</td>
<td>93.2%</td>
<td>6.8%</td>
<td>2021</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Fertility was assessed by counting the number of aborted seeds in mature siliques. This evaluation was performed in 10, 10 and 7 plants for pdx1.1-1, pdx1.3 and wild-type, respectively. No significant difference was observed for pdx1.1-1 (\(P > 0.05\)) when compared to wild type plants. In contrast, a significant reduction in the number of fertilized seeds was observed in pdx1.3 (\(P < 0.01\)).

Figure 3. Phenotypes of pdx1.1 and pdx1.3. (a) Comparison of 17-day old wild-type, pdx1.1-1, pdx1.1-2 and pdx1.3 plants grown in soil. The developing leaves of pdx1.3 are slightly chlorotic. (b) Wild-type (middle), pdx1.1-1 (left) and pdx1.3 (right) plants after 35 days of growth in soil. (c) Growth of wild-type, pdx1.1-1, pdx1.1-2 and pdx1.3 after 9-days on sterile medium in plates maintained in a vertical position, in the absence (-) and presence (+) of pyridoxine (5 µM). In pdx1.3, shoot and root development is strongly retarded. The delay in root development is much less pronounced in pdx1.1-1 and pdx1.1-2.
Figure 4. Nine-day old wild-type, pdx1.1, pdx1.3, PDX1.1/pdx1.1 pdx1.3/pdx1.3 and pdx1.1/pdx1.1 PDX1.3/pdx1.3 seedlings grown in sterile culture under 16 hours of 100 µmol photons m$^{-2}$ s$^{-1}$ day$^{-1}$ of white light in the absence or presence of pyridoxine (5 µM).

3.2.4 Phenotypes of the *pdx1.1 pdx1.3* double mutant can be correlated with the vitamin B6 content

As *pdx1.1-1* and *pdx1.1-2* appeared to have identical phenotypes all further experiments described were carried out with only one mutant line, *pdx1.1-1*, and will be referred to simply as *pdx1.1* from now on. In an attempt to assess the effect of complete removal of the functional PDX1s, reciprocal crosses were performed of *pdx1.1* and *pdx1.3*. Heterozygous double-mutant plants from the F$_1$ generation were allowed to self-fertilize and the resulting progeny were analyzed. No homozygous double-mutant plants were recovered based on PCR genotyping. To further investigate the absence of offspring homozygous for the knockout of both genes, siliques from plants that were heterozygous for *pdx1.1* and *pdx1.3* from each reciprocal cross were compared to wild-type. In each case, a 1:15 segregating population of albino:green seeds could be observed in the *PDX1.1/pdx1.1 PDX1.3/pdx1.3* plants (Figure 6a and Table 3), representing the *pdx1.1 pdx1.3* population.
A histological analysis of different developmental stages indicated that the *pdx1.1 pdx1.3* seeds were developmentally impaired compared to their siblings. At all stages examined approximately 1 out of 16 seeds did not progress from the globular stage of embryo development (Figure 6b), indicating arrested growth of *pdx1.1 pdx1.3* seeds. The development of *PDX1.1/pdx1.1 PDX1.3/pdx1.3* plants was indistinguishable from that of the wild-type (data not shown).

Figure 5. Vitamin B6 and chlorophyll content. (a) Chlorophyll content of leaf 9 of 27-day old plants in soil. The results shown are the mean values and standard deviation of three independent experiments. (b) Vitamin B6 content of 28-day old plants grown in soil. (c) The same lines as in (b) but grown in sterile medium in the absence (white bars) and presence (black bars) of pyridoxine (5 µM). Seedlings were 14-days old. In the case of (b) and (c), the vitamin B6 content of each line, *pdx1.1, pdx1.3, PDX1.1/pdx1.1 pdx1.3/pdx1.3* and *pdx1.1/pdx1.1 PDX1.3/pdx1.3* is expressed as a percentage of that in wild-type (Col-0) grown in soil, in the presence or absence of pyridoxine (3.2, 3.0 and 2.5 ng mg⁻¹ FW, respectively). The results are the mean values and standard deviation of three independent experiments, each of which was performed in triplicate from the rosette leaves of four plants.
However, we observed distinctions in the development of plants that carry only a single functional copy of either PDX1.1 i.e. PDX1.1/pdx1.1 pdx1.3/pdx1.3 or PDX1.3 i.e. pdx1.1/pdx1.1 PDX1.3/pdx1.3. In soil, the aerial parts of single copy PDX1 plants are developmentally retarded compared to wild-type (Figure 7a). Furthermore, in both genotypes the youngest leaves are strikingly chlorotic (Figure 7a), as is also apparent from the chlorophyll measurements (Figure 5a). As the leaf develops, a gradient of green color is observed extending from the leaf tip. These features are strongest in single copy PDX1.1 plants i.e. that do not carry a functional copy of PDX1.3 (Figure 7a) and furthermore, are most evident in young seedlings. Interestingly, although single copy PDX1.3 plants are strongly delayed in development, they perform the switch from vegetative to reproductive growth at approximately the same time as the wild-type (Figure 7b,d). Thus, at the time of the switch, these plants have rosette leaves that are severely reduced in size compared to wild-type (Figure 7c). On the other hand, while plants that have a single copy of PDX1.1 are even more delayed than single copy PDX1.3 plants (Figure 7b), they proceed through development producing rosette leaves indistinguishable from those of wild-type plants. Only once the vegetative stage is complete do they make the switch to the reproductive stage. Both sets of single copy PDX1 plants proceed through the reproductive stage, albeit at a severely retarded rate, developing normal sized siliques (Figure 6c). Analysis of immature siliques of both genotypes revealed a somewhat heterogeneous phenotype in that between 21-34% had seeds which were either albino or severely malformed and later aborted, or had non-fertilized ovules (Figure 6a).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Seed abortion in pdx1 mutant plants</th>
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<tbody>
<tr>
<td>Plant</td>
<td>Normal seeds</td>
</tr>
<tr>
<td>PDX1.1/pdx1.1</td>
<td>94.36%</td>
</tr>
<tr>
<td>PDX1.3/pdx1.3</td>
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</table>

Mature siliques from PDX1.1/pdx1.1 PDX1.3/pdx1.3 plants were analyzed for the presence of albino seeds. A $P$ value of > 0.05 was considered consistent with the hypothesis that 6.25% of the seeds abort as expected for a zygotic embryonic lethal mutation.

The phenotypical differences between single copy PDX1.3 and PDX1.1 became more evident on sterile culture in the absence of vitamin B6 where the majority of a segregating population of single copy PDX1.1 seedlings did not develop beyond the cotyledon-primary leaf stage (Figure 4). The addition of 5 µM pyridoxine to the medium restored growth to that observed with the wild-type under the same conditions. We also measured the vitamin B6 content of single copy PDX1 plants, both in soil and in sterile culture, and in the absence and presence of the vitamin (Figure 5). A correlation is found between the phenotype observed and the vitamin B6 content,
in that the most severe growth impairment is observed when the vitamin B6 content is lowest, which in turn is when PDX1.3 is absent. This is most evident when plants are grown in the absence of a vitamin B6 supplement (Figure 5b,c).

![Figure 6. Silique phenotype of heterozygous/homozygous double mutants.](image)

(a) Immature silique of wild-type (top panel) and plants heterozygous for both or one of either pdx1.1 or pdx1.3. The albino phenotype of pdx1.1 pdx1.3 (one sixteenth) can be observed (black arrowheads) in PDX1.1/pdx1.1 PDX1.3/pdx1.3 (second panel). Developing silique of PDX1.1/pdx1.1 pdx1.3/pdx1.3 (third panel) and a more mature pdx1.1/pdx1.1 PDX1.3/pdx1.3 silique (bottom panel) where a combination of incomplete fertilization and aborted seeds could be scored. (b) Cleared whole mounted seeds from PDX1.1/pdx1.1 PDX1.3/pdx1.3 showing representative examples of either a wild-type, single or double heterozygote at heart stage (top), and a pdx1.1 pdx1.3 seed (bottom) indicating arrest at the globular stage of embryo development. Both seeds were from the same silique and were viewed with Nomarski optics. The scale bar represents 100 µm. (c) The left panel shows the oldest whole siliques from wild-type, pdx1.1, pdx1.3 and pdx1.1/pdx1.1 PDX1.3/pdx1.3, respectively, after 45 days. The right panel shows the youngest silique from pdx1.1/pdx1.1 PDX1.3/pdx1.3 and the oldest from PDX1.1/pdx1.1 pdx1.3/pdx1.3 after 78-days. The scale bar represents 5 mm.
Figure 7. Shoot phenotype of homozygous/heterozygous double mutants. (a) Comparison of 17-day old single and double knockouts grown in soil. No pdx1.1/pdx1.1 pdx1.3/pdx1.3 plants were identified. Both pdx1.1/pdx1.1 PDX1.3/pdx1.3 and PDX1.1/pdx1.1 pdx1.3/pdx1.3 are retarded in shoot growth in addition to the leaves being highly chlorotic. The phenotype is most pronounced in PDX1.1/pdx1.1 pdx1.3/pdx1.3. (b) Wild-type, pdx1.1/pdx1.1 PDX1.3/pdx1.3 and PDX1.1/pdx1.1 pdx1.3/pdx1.3 plants after 35 days of growth in soil. (c) Rosette leaves of pdx1.1/pdx1.1 PDX1.3/pdx1.3 plants are impaired in expansion. In the order from left to right, the same number leaf from 35-day old wild-type, pdx1.1, pdx1.3, PDX1.1/pdx1.1 pdx1.3/pdx1.3 and pdx1.1/pdx1.1 PDX1.3/pdx1.3 plants, respectively. (d) Percentage of plants that have bolted after 24 (black), 31 (light grey), 38 (dark grey) and 45 (medium grey) -days of growth.

3.2.5 PDX1.3 deficiency increases salt sensitivity and photo-oxidative stress

It has recently been shown that PDX1.3 is required for plant adaptation to salt, osmotic and oxidative stress, respectively (Chen and Xiong, 2005). In this context and with both pdx1.1 and pdx1.3 knockout plants at hand, an analogous investigation was performed to assess if there is a distinction between the absence of either PDX1.1 or PDX1.3 in the response to stress. Seeds of wild-type and mutant lines were germinated and grown for seven days on sterile medium supplemented with vitamin B6, such that they were virtually indistinguishable from each other (Chen and Xiong, 2005). The seedlings were then transferred to medium without vitamin B6 supplementation and in the presence of either sodium chloride or mannitol. We observed that while pdx1.3 was hypersensitive to both salt and osmotic stress, pdx1.1 sensitivity appeared to be similar to that of the wild-type (Figure 8).
Figure 8. Sensitivity of *pdx1.1* and *pdx1.3* mutant seedlings to abiotic stress. Morphology of 28-day old wild-type, *pdx1.1* or *pdx1.3* seedlings, respectively, on MS plates lacking vitamin B6 and in the presence of either sodium chloride (100 mM) or mannitol (100 mM). Seedlings were first grown for 7-days on MS plates containing pyridoxine (100 µM) before being transferred to the plates shown.

Interestingly, as our expression analysis showed that both *PDX1.1* and *PDX1.3* are regulated by light (Figure 1c), this prompted us to investigate whether the observed phenotypes would be affected by light. Firstly, wild-type, *pdx1.1* and *pdx1.3* plants were grown on MS medium without pyridoxine, under moderate light intensity (100 µmol photons m⁻² s⁻¹) and chlorophyll a fluorescence measurements were performed on nine-day old seedlings. Under these conditions, the maximum quantum efficiency of photosystem II (PSII) photochemistry ($F_v/F_m$) was reduced from 0.86 in wild-type to 0.72 in *pdx1.3* plants (Student’s t-test p-value <0.0001) indicating a significant photoinhibition of PSII in *pdx1.3* (Figure 9a). Conversely, when the plants were grown under low light (10 µmol photons m⁻² s⁻¹), there was no significant effect on PSII photochemistry ($F_v/F_m$ equal to 0.84 in wild-type and 0.83 in *pdx1.3*, Student’s t-test p-value = 0.37). Furthermore, under these conditions, chlorosis of *pdx1.3* was no longer observed in sterile culture, and *pdx1* mutants were indistinguishable from wild-type (compare Figure 9c to Figure 4). To further assess the light sensitivity of *pdx1* mutants, plants grown in low light were exposed to photoinhibitory conditions. Nine-day old seedlings were exposed to high light (1100 µmol photons m⁻² s⁻¹) over a period of several hours. This treatment caused a rapid inhibition of PSII photochemistry, with the $F_v/F_m$ decreasing by 20% after 1 hour in wild-type (Figure 9b). PSII photoinhibition was more rapid and pronounced in *pdx1.3*, $F_v/F_m$ decreased by 29% after 1 hour.
(35% after 2 h, 27% in wild type). pdx1.1 seedlings showed a similar decline of $F_v/F_m$ to that of wild type albeit slightly accelerated (Figure 9b).

Figure 9. Effect of light intensity and photoinhibitory treatments on wild-type, pdx1.1 and pdx1.3. (a) Maximum quantum efficiency of PSII photochemistry ($F_v/F_m$) of wild-type and pdx1 mutant lines grown under 100 or 10 µmol photons m$^{-2}$ s$^{-1}$. Results are the mean values and standard deviation of 40 to 60 measurements from 9-day old seedlings. (b) Photoinhibitory treatments of wild-type and pdx1 mutant lines grown under 10 µmol photons m$^{-2}$ s$^{-1}$ and then exposed to 1100 µmol photons m$^{-2}$ s$^{-1}$, respectively. Each point is the result of 40 to 60 measurements from 9-day old seedlings. (c) Nine-day old wild-type and pdx1.1 and pdx1.3 mutant lines grown in sterile culture under 10 µmol photons m$^{-2}$ s$^{-1}$ of white light in the absence of vitamin B6. (d) Immunochemical analysis of the D1 and LHC protein content in 12-day old seedlings of wild-type, pdx1.1 and pdx1.3 grown in the absence and presence of vitamin B6 under either 100 or 10 µmol photons m$^{-2}$ s$^{-1}$, respectively, of white light.

During high light stress, $^1$O$_2$ is produced from chlorophyll excitation in the light harvesting complexes (LHC) or the PSII reaction centers. Photoinhibition is related to the rapid turnover of the D1 protein, and $^1$O$_2$ produced within PSII is a probable intermediate in the triggering of the degradation of this protein. The steady state level of the D1 protein was analyzed in wild-type
and *pdx1* seedlings grown in the presence and absence of vitamin B6 under 10 and 100 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) of light. While the D1 protein could be detected under all conditions and in all seedlings examined (Figure 9d), it was substantially reduced in *pdx1.3* seedlings grown in the absence of vitamin B6 under moderate light conditions. Growth of *pdx1.3* seedlings in the presence of 5 \( \mu \)M pyridoxine or under low light restored the D1 level to that approaching wild-type (Figure 9d). In contrast, no significant difference in the levels of the LHC could be detected between the samples examined (Figure 9d).

### 3.3 Discussion

From this and other recent studies, it is becoming increasingly apparent that vitamin B6 performs multiple roles in plants. It is well established that the vitamin is required for normal cellular functions, because in its cofactor form(s), PLP or PMP, it is necessary for numerous metabolic reactions. However, the vitamin can also function as an antioxidant (Ehrenshaft et al., 1999; Jain and Lim, 2001), a role which has recently been demonstrated in plants. For example, supplementation with the pyridoxine vitamer in one case reduced singlet oxygen-induced cell death in *Arabidopsis* (Danon et al., 2005) and in another study, it delayed ROS-dependent pathogen defense responses in *Nicotiana tabacum* (Denslow et al., 2005). This implies that a balance has to be achieved *in vivo* where the functionality of the vitamin within the cell i.e. as a cofactor and/or antioxidant, is most likely fulfilled through maintenance of “vitamin B6 homeostasis”. This could be facilitated by the *de novo* and/or salvage pathways of biosynthesis, both of which are known to exist in plants (Shi and Zhu, 2002; Tambasco-Studart et al., 2005).

#### 3.3.1 The *de novo* biosynthesis pathway is the predominant source of vitamin B6 in plants

While the salvage pathway of vitamin B6 biosynthesis has been established in most organisms, the predominant *de novo* biosynthesis pathway has only recently been deciphered (Burns et al., 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005). In the *de novo* pathway, the two proteins, PDX1 and PDX2 function together as a glutamine amidotransferase, with PDX2 as the glutaminase domain and PDX1 as the synthase domain, resulting in the direct formation of the cofactor vitamer, pyridoxal 5’-phosphate (Burns et al., 2005; Raschle et al., 2005). There are three PDX1 and one PDX2 homolog(s) in *Arabidopsis*. Here, we have shown that disruption of the two functional PDX1 paralogs, PDX1.1 and PDX1.3 in *Arabidopsis*, leads to an embryo-lethal phenotype. The arrest in embryo development is remarkably similar to what
was observed by knocking out the single copy PDX2 (Tambasco-Studart et al., 2005). The fact that knocking out both PDX1.1 and PDX1.3 is lethal for the plant supports our previous analyses indicating that PDX1.2 does not catalyze biosynthesis of PLP in Arabidopsis (Tambasco-Studart et al., 2005) and therefore, cannot compensate for the absence of PDX1.1 and PDX1.3. The role of the PDX1.2 protein has yet to be deciphered, and while it is more divergent compared to PDX1.1 and PDX1.3, our own analysis (data unpublished) and that deciphered from Genevestigator (Zimmermann et al., 2004) indicate a very different mode of regulation (Figure S1). Moreover, we have observed that amino acids thought to be involved in catalysis by PDX1 (Zhu et al., 2005) are not conserved in PDX1.2.

The failure to identify seedlings homozygous for either pdx1.1 pdx1.3 or pdx2 (Tambasco-Studart et al., 2005) implies that the salvage pathway cannot compensate for the loss of the de novo biosynthesis pathway. This further implies that the de novo pathway is essential for plant viability and that it plays the dominant role in maintaining vitamin B6 homeostasis. This is corroborated by the observations with the single and double homo/heterozygote pdx1.1 pdx1.3 plants, because even when the de novo pathway is merely compromised, the plants show developmental impairments. Furthermore, it has been shown that the PL kinase, SOS4, is not essential for plant viability (Shi et al., 2002; Shi and Zhu, 2002). This mutant is impaired in root growth. Thus, we were somewhat surprised that in comparison to other organs, the PDX1.1 or PDX1.3 genes are not highly expressed in the root. This could be explained if vitamin B6 is not directly involved in promoting root growth and development but rather has an indirect role, affecting vitamin B6 dependent enzymes which control the synthesis of compounds known to regulate the process e.g. tryptophan, auxin (Casimiro et al., 2003). It is interesting, that the phenotype of the mutant plants analyzed is much less pronounced in soil, as compared to sterile medium in the absence of vitamin B6 (compare Figures 3, 4 and 7). In this context, it is perhaps significant that the pdx1 phenotype can be rescued in sterile medium by supplementation with pyridoxine as shown. This implies that in Arabidopsis there is an uptake system in place for the vitamin. Thus, one explanation for the less severe phenotype in soil might be that there is a source of the vitamin within the soil, the nature of which is currently not known. We observed that in sterile culture pyridoxal could also rescue the pdx1 phenotype (unpublished observations). Therefore, it is likely that in addition to the characterized PL kinase, SOS4 (Shi and Zhu, 2002), there are presumably PN/PM oxidases and/or kinases that are important components of the vitamin B6 salvage pathway in Arabidopsis.
3.3.2 Evidence for redundancy between \textit{PDX1.1} and \textit{PDX1.3}

The predominance of \textit{PDX1.3} expression compared to \textit{PDX1.1} can be correlated with the more severe phenotype in \textit{pdx1.3} plants (particularly in sterile culture) and the corresponding reduced vitamin B6 content indicative of a dosage effect. Intriguingly then, a comparison of \textit{pdx1.1/pdx1.1 PDX1.3/pdx1.3} with \textit{PDX1.1/pdx1.1 pdx1.3/pdx1.3} suggests that there is a threshold, apparently mediated by the level of \textit{PDX1.3}, involved in the control of the switch from vegetative to reproductive (V-R) growth. While a delay in shoot growth is observed in plants that carry only a single copy of \textit{PDX1.3}, the V-R switch occurs at a similar time to wild-type plants. Thus, at the time of the switch the rosette leaves are at an intermediate stage where they have not yet fully expanded. This is in contrast to single copy \textit{PDX1.1} plants, i.e. do not carry a functional copy of \textit{PDX1.3}, where development is very severely delayed, but proceeds through the growth phases until the mass of vegetative tissue reaches a similar level to that observed in wild-type. Only then does the V-R switch occur. It is well documented that the plant switches to flowering at a time when sufficient internal resources have been accumulated (Simpson et al., 1999). In this context, it could be that the vitamin B6 content may be an important resource for reproductive development sensed by the plant.

3.3.3 Vitamin B6 plays a role in the oxidative stress response

In this study we show that plants, with a reduced vitamin B6 content are hypersensitive to salt, osmotic and light stress. High soil salinity and increased osmolarity can result in the accumulation of reactive oxygen species (Xiong et al., 2002; Mazel et al., 2004). In addition, photosynthesis continuously produces reactive oxygen species (ROS) inside the chloroplast that are kept under control by both enzymatic and non-enzymatic scavenging systems (for a recent review see (Apel and Hirt, 2004)). During high light stress, the production of ROS dramatically increases, in particular \textit{1O}_2, which is generated from chlorophyll excitation in either the light harvesting complexes and/or the PSII reaction centers. Some of the B6 vitamers have been shown to be potent singlet oxygen quenchers \textit{in vitro} (Bilski et al., 2000). This has also been demonstrated \textit{in vivo} in the necrotrophic fungus \textit{Cercospora nicotianae} (Ehrenshaft et al., 1998), where it protects against the photosensitizer toxin, cercosporin that kills plants primarily via \textit{1O}_2-mediated peroxidation of membrane lipids (Daub, 1982; Daub and Ehrenshaft, 2000), and as mentioned above in the conditional \textit{flu} mutant of \textit{Arabidopsis} (Danon et al., 2005). In plants, the quenching of \textit{1O}_2 has been linked to the turnover of the D1 protein so that decreased accumulation of the protein in PSII is thought to be an indicator of photoinhibition due to \textit{1O}_2 (Aro et al., 1993; Hideg et al., 1998; Havaux et al., 2005). Therefore, in the absence of other \textit{1O}_2...
quenching mechanisms, the destruction of D1 is essential to detoxify $^{1}$O$_{2}$ directly at the place of its generation and prevent damage of PSII (Aro et al., 1993). Thus, the drastically reduced amount of D1 and apparent lack of effect on the LHC observed with pdx1 suggests that in these mutant plants the $^{1}$O$_{2}$ concentration is increased in the PSII reaction centre and that vitamin B6 is important to control D1 degradation and PSII integrity by detoxifying $^{1}$O$_{2}$ produced in PSII as has been proposed for vitamin E (Havaux et al., 2005).

Damage of PSII was assessed in this study by measuring the maximum quantum efficiency of PSII photochemistry ($F_{v}/F_{m}$). pdx1.3 and to a much lower extent pdx1.1 seedlings grown under moderate light (100 µmol photons m$^{-2}$ s$^{-1}$) had a $F_{v}/F_{m}$ lower than in wild-type seedlings, indicating a considerable photoinhibition that correlates with the decreased amounts of the D1 protein. However, under low light both the $F_{v}/F_{m}$ and the D1 level are very similar to wild type values suggesting that moderate light is already a constant stress for pdx1 mutants. Therefore, vitamin B6 must have an important photoprotective role even under moderate light. This is corroborated by the ability of exogenous pyridoxine to restore D1 accumulation. This implies that a fraction of the vitamin B6 pool must be located very close to PSII for efficient quenching of $^{1}$O$_{2}$ at its site of production and for prevention of D1 oxidation, although the intracellular location of vitamin B6 has not yet been examined. The more sensitive response of pdx1.3 compared to pdx1.1 could be explained by the higher concentration of vitamin B6 in the latter. However, vitamin B6 is also most likely required as a cofactor for some of the enzymes involved in chlorophyll biosynthesis, notably, glutamate 1-semialdehyde aminotransferase and uroporphyrinogen decarboxylase. Therefore, we cannot rule out the possibility that the photosensitivity of the pdx1 mutants might originate from a chlorophyll turnover deficiency as well as from a deficiency in $^{1}$O$_{2}$ quenching.

In this study, we have demonstrated that both PDX1.1 and PDX1.3 are necessary for plant viability. We have shown that compromising the de novo pathway of vitamin B6 biosynthesis results in both a developmental and stress sensitive phenotype. The latter observation would appear to indicate that PLP plays a protective role against reactive oxygen species in vivo, in addition to its function as a cofactor. However, it remains to be elucidated whether this is as a prerequisite for another enzyme involved in regulation of oxidative stress or as a substrate for enzymes of the vitamin B6 salvage pathway. The availability of single and double mutations in the de novo pathway and the possibility to identify and characterize further mutants in the salvage pathway provides the opportunity to test hypotheses proposed and furthermore to investigate the regulation of vitamin B6 homoeostasis.
3.4 Experimental Procedures

3.4.1 Plant material and growth conditions

Arabidopsis thaliana (wild-type, ecotype Col-2 or Col-0, depending on the experiment; SM_3_22664; GABI_423C02; SALK_086418 and their progeny) were grown in soil under a 16-hour-day (100 µmol photons m\(^{-2}\) s\(^{-1}\) of light intensity) and 8-hour-night cycle at 22°C/19°C, respectively. For the tissue expression analysis, roots, stems, flowers, siliques, rosette leaves and cauline leaves were collected from at least ten plants. Cotyledons were collected at 2-weeks from seedlings grown under the same conditions. For the developmental expression analysis, samples from rosette leaves were collected over a period of 2 to 11-weeks. When grown in sterile culture, Arabidopsis seeds were first surface-sterilized, plated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1% w/v sucrose and 0.9% w/v agar and kept for 4 days in the dark at 4°C after which the plates were transferred to continuous light (100 µmol photons m\(^{-2}\) s\(^{-1}\) or 10 µmol photons m\(^{-2}\) s\(^{-1}\) as specified) at 22°C. In the case of the light induction analysis, plates were transferred to continuous dark at 20°C for 5 days. The etiolated seedlings were then transferred to light at an intensity of 150 µmol photons m\(^{-2}\) s\(^{-1}\) and whole plantlets were collected after 15 minutes, 1 hour, 5 hours and 24 hours of exposure to light. Complementation experiments were performed by growing seedlings on MS plates under the same light and temperature conditions, either in the absence or presence of pyridoxine or pyridoxal (5 µM). For the analysis of stress sensitivity, seedlings were grown for seven days after imbibition of the seeds on plates containing 100 µM pyridoxine, after which they were transferred to plates lacking the vitamin, and either in the presence or absence of sodium chloride or mannitol (100 mM). In all cases, samples of plant material collected were immediately frozen in liquid nitrogen and maintained at -80°C until further use.

3.4.2 Isolation of RNA and quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen AG, Switzerland) and treated with RNase-free DNase (Qiagen) to remove traces of DNA, according to the manufacturer’s instructions. First strand cDNA synthesis was performed at 42°C for 60 minutes in a volume of 20 µl, containing 1 or 2 µg of total RNA, 20 pmol of oligo(dT) and 200 units of reverse transcriptase from the Advantage RT-for-PCR kit (Takara Bio Europe/Clontech, Switzerland). For transcript quantification, the equivalent of 50 ng of total RNA was employed. Quantification analyses were performed using an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA), by fluorescence-based real-time PCR with the fluorescent dye SYBR Green. The PCR conditions for all genes analyzed were as follows: an initial
denaturation at 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Relative mRNA abundance was calculated using the comparative ΔCt method and normalized against the constitutively expressed actin-2 (At3g18780) or ubiquitin (At5g25760) gene, respectively. The primers used for this quantification were: PDX1.1 forward, 5’-GTGAGGAGTGTGAACGGAGC-3’; PDX1.1 reverse, 5’-GCACAACCATAATCATACGCGC-3’; PDX1.2 forward, 5’-GATGCAGCTAGGTTGTGTGATGG-3’; PDX1.2 reverse, 5’-TCCATTGCATTCTCCAATCC-3’; PDX1.3 forward, 5’-ATAATTTCGGATCCCGTGTC-3’; PDX1.3 reverse, 5’-CATCATCATCATCCATGTTCGC-3’; Actin-2 forward, 5’-ATTCTTGCTTCCCTCAGCAC-3’; Actin-2 reverse, 5’-CCCCAGGTITTAAGGCTTTT-3’. The control primers for the Arabidopsis ubiquitin gene were from Sigma-Aldrich (Buchs, Switzerland).

3.4.3 Immunochemical analyses

For Western blot analysis, proteins were extracted from frozen plant material by homogenization in liquid nitrogen using a mortar and pestle and 1 volume of 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA and protease inhibitor cocktail. Debris was removed by centrifugation at 10,000 g for 15 min at 4°C. Total protein was quantified using a Coomassie protein assay kit (Socochim, Lausanne, Switzerland) and was separated by 11% SDS-polyacrylamide gel electrophoresis (30 µg per lane). For immunodetection, the protein was blotted onto a nitrocellulose membrane and blocked by incubation with Tris Buffered Saline (TBS, 10 mM Tris-HCl, pH 7.5, 0.9% sodium chloride) containing 0.05% Tween-20 and 5% milk powder. PDX1 was detected by incubating the blots for 3 hours with an antibody raised against the homologous Bacillus subtilis Pdx1 protein in rabbit, and which had been affinity purified before use against both PDX1.1 and PDX1.3 (1:1000 dilution). This was followed by incubation for 45 min with a 1:3000 dilution of a goat anti-rabbit peroxidase conjugated secondary antibody (BioRad, Hercules, CA) for chemiluminescent detection. The light-harvesting-complex (LHC) was detected in an analogous fashion using a 1:10,000 dilution of a specific antibody (AgriSera, Vännäs, Sweden). The D1 protein was observed using anti-PsbA Global (AgriSera, Vännäs, Sweden) at a 1:1500 dilution for 3 hours followed by incubation with a 1:5000 dilution of anti-chicken IgG peroxidase conjugate (Sigma-Aldrich, Buchs, Switzerland) for 1 hour.

3.4.4 Generation of pdx1.1 pdx1.3 mutant plants

Plants homozygous for pdx1.1-1 were crossed with those homozygous for pdx1.3 to generate the double pdx1.1 pdx1.3 insertion mutants. The F1 progeny heterozygous for each of
the *pdx1* insertions was allowed to self fertilize to obtain the F₂ population in which plants homozygous for either one of the *pdx1* insertions and heterozygous for the other were identified. No seedlings homozygous for both *pdx1.1* and *pdx1.3* were identified. Progeny from the F₃ generation was used in the experiments described.

### 3.4.5 Analysis of mutants by PCR

Genomic DNA of the *Arabidopsis* T-DNA lines (GABI_423C02, SALK_086418), the SM_3_22664 transposant, or the progeny of crosses was extracted and screened for the T-DNA or *Ds* transposon insertions at the *PDX1.1* or *PDX1.3* loci. Forward and reverse primers from the sequences of the *PDX1.1* or *PDX1.3* genes were designed for PCR screening with a combination of T-DNA left or right border-specific (Alonso et al., 2003) or *Ds*-5' or *Ds*-3' primers (Tissier et al., 1999). Flanking sequences of insertion loci were confirmed by sequencing. Sequences of primers used are as follows: *PDX1.1*-F, 5' CGGGACTATACATTACAACAAAG-3' and *PDX1.1*-R 5' CCAAGTATTATCTGTATTCCCTGTC-3'; *PDX1.3*-F, 5' CAAATTCTCTTCAATCTCCG-3' or SORL1-F1, 5' GGTAACGGTGCGATAACGG-3' and *PDX1.3*-R, 5' GGATATCAGCAGAACAGC-3'; *Ds*-5', 5' CTTATTTCAGTAAGAGTGTTGGGTTTT-GG-3'; GABI T-DNA 8409 (Rosso et al., 2003), 5' ATATTGACCGGCTCGCG-3'; SALK T-DNA LB primer (http://www.salk.edu/), 5' GCYGTGACCCTTTGCTGCAACT-3'. PCR conditions were as follows: denaturation at 94°C for 2 min, then 35 cycles of 45 s at 94°C, 45 s at 57°C, and 1 min at 72°C, followed by 5 min at 72°C.

### 3.4.6 DNA gel blot analysis

Genomic DNA (4 µg) from individual plants was digested overnight with the selected restriction enzymes. The resulting fragments were separated on 0.8% agarose gels for 5 h at 80 V and transferred to Hybond-N membrane (Amersham Biosciences, UK). DIG labeled probes were generated according to the manufacturer's instructions (Roche Diagnostics, Germany) using the following primers: *BAR* gene forward, 5'-TCATCAGATCTC-GGTGACGGGCAG-3' and reverse 5'-CATGAGCCCAGACGCCCAGG-3' using pSLJ 8313 as template (kindly provided by the SINS laboratory); *NPTII* gene forward, 5'-GACGACTCATTTTTTCTTGCAACT-3' and reverse 5'-GGAGCTCGTCGACGGC-3' using pROK2 as template. Membranes were pre-hybridized according to the manufacturer's instructions employing 3 µl of probe per ml of DIG Easy hybridization buffer. Stringent washes were used to achieve an allele-specific hybridization signal. For the chemiluminescent detection, the substrate CDP-Star (Roche...
Diagnostics, Germany) was used. In all cases a consistent number of bands was obtained between individual plants.

3.4.7 Measurement of chlorophyll content

Chlorophyll was extracted from individual rosette leaves with 100% acetone. Chlorophyll content was determined spectrophotometrically from the absorbance at 663 nm and 646 nm according to (Lichtenthaler, 1987).

3.4.8 Fertilization rate

The fertilization rate was calculated by analyzing dissected siliques and scoring fertilized versus unfertilized embryos.

3.4.9 Whole mount preparation of ovules

Siliques at different developmental stages were harvested, dissected under a stereoscope, and fixed overnight in an ethanol/acetic acid (3:1 v/v) mixture at 4°C followed by rehydration in ethanol and water (3:1 v/v). Fixed siliques were cleared according to a protocol described by (Tambasco-Studart et al., 2005) in chloral hydrate/glycerol/water (20:2:5 w/w/w) for 24 to 48 hours at 4°C. Cleared ovules were further removed from their siliques in a drop of the same clearing solution, whole-mounted, and observed using a Zeiss Axioplan microscope (Zeiss, Jena, Germany) equipped with AxioCam HRc camera and Normaski optics. Digital images were captured using Axiovision 3.0 software (Zeiss).

3.4.10 Vitamin B6 bioassay

Samples for vitamin B6 analysis were obtained either by growing seedlings in sterile medium in the presence or absence of pyridoxine, or were from rosette leaves of plants grown in soil. In each case samples were harvested, frozen in liquid nitrogen and stored at -80°C until analysis. The amount of vitamin B6 in the collected samples was determined by employing the microbiological assay as described in (Tambasco-Studart et al., 2005). The vitamin was extracted from the plant material (2-5 mg) by homogenization with a mortar and pestle in 0.02 M H$_2$SO$_4$, after which the sample was autoclaved at 121°C for at least 1 hour. After extraction, the solution was neutralized to pH 5.2, using sodium acetate and centrifuged for 15 minutes at 10,000 g to remove debris. Levels of vitamin B6 were quantified by extrapolation to a standard
curve obtained from growth of the *Saccharomyces carlsbergensis* ATCC 9080 strain in the presence of pyridoxine. All samples and standards were determined in triplicate.

### 3.4.11 Measurements of maximum quantum efficiency of PSII photochemistry ($F_v/F_m$)

$F_v/F_m$ was measured after 15 minutes dark adaptation with a FluorCam (Photon Systems Instruments, Czech Republic). Nine day-old seedlings of wild-type and *pdx1* mutant lines grown on MS medium without pyridoxine and under continuous light at either 100 or 10 µmol photons m$^{-2}$ s$^{-1}$, were exposed to either the same light intensity or 1100 µmol photons m$^{-2}$ s$^{-1}$ for one to 6 hours prior to chlorophyll a fluorescence measurements.

### 3.5 Acknowledgments

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**Abbreviations:** DD, continuous dark; DXP, deoxyxylulose 5-phosphate; EST, expressed sequence tag; LHC, light harvesting complexes; LL, continuous light; PL, pyridoxal; PLP, pyridoxal 5’-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5’-phosphate; PN, pyridoxine; PNP, pyridoxine 5’-phosphate; WT, wild-type.
3.6 Supplemental Material

Figure S1. Relative expression of *Arabidopsis PDX1* genes at different developmental stages, in various tissues and in response to various forms of stress. *Gene Correlator* was used to plot the expression of two *PDX1* genes over the 2204 GeneChips available in the *Genevestigator* microarray database (Zimmermann et al., 2004). *PDX1* genes plotted on the X and Y axes are indicated. Each spot represents one individual GeneChip. Red spots: both genes “present” on the chip, i.e. both genes have a detectable level of expression (*p*-values < 0.05); green spots: both “absent” (*p*-values >= 0.05); dark blue: gene on the X axis is present, gene on the Y axis is absent; light blue: gene on the Y axis is present, gene on the X axis is absent.

Figure S2. Southern blot analyses of *pdx1.1-1* and *pdx1.3*. Genomic DNA was extracted from wild-type (WT) and the respective knock out plants, digested with the indicated restriction enzymes and used for Southern blot analysis. In each case, a specific DIG labeled probe was used to detect the digested fragments. (a) SM_3_22664 line; in this case a probe specific to the phosphinothricin acetyltransferase gene (*BAR*) was used to detect *Xma*I and/or *Hind*III digested fragments. The pattern reveals a single insertion on the *PDX1.1* locus. (b) SALK_086418 line, *EcoR*I and/or *Hind*III fragments were detected employing a probe specific to the kanamycin
resistance gene (NPTII). The pattern reveals that there are three T-DNA insertions at the PDX1.3 locus.

Figure S3. Development of roots in *pdx1.3* versus wild-type. (a) Development of wild-type versus *pdx1.3* in sterile culture indicating that while *pdx1.3* shows a severe delay in shoot and root growth, it does proceed. A comparison of *pdx1.3* after 15 days with the wild-type at 7-days reveals that the retardation of *pdx1.3* growth is more pronounced for the root rather than the shoot. (b) Elongation rates of primary roots in *pdx1.3*, *pdx1.1-1* and *pdx1.1-2* mutants versus the wild-type plants. Four-day old seedlings grown vertically on MS medium without pyridoxine were marked at the root tips and the extent of new root growth was measured after 3 days. Error bars represent standard deviations.
3.7 References


4  **PDX1.1 and PDX1.3 phytohormonal regulation**

**Introduction**

4.0.1 **How phytohormones regulate plant development in general**

In chapter 3 of this thesis and by other independent parallel studies, it has been shown that PDX1.1- and PDX1.3-deficient *A. thaliana* plants display a number of developmental discrepancies (Chen and Xiong, 2005; Wagner et al., 2006; Titiz et al., 2006). In order to gain some insight and understanding of the possible factors which could result in such developmental discrepancies, it is important to understand how plant growth and development, in general, are regulated. The development of multi-cellular organisms such as plants from a single cell requires the correct spatial and temporal expression of genes, coordinated by signalling systems. For many years, insight into intercellular signalling in plants was based on knowledge of the so-called classical plant hormones, namely ethylene, auxin, cytokinin, gibberellin, and abscisic acid. It was assumed that these classical hormones each regulate some part of development through individual signalling pathways (Guern, 1987).

The plant hormone ethylene has wide ranging and dramatic effects on the growth, development and stress responses of the plant throughout its life (Abeles et al., 1992). Ethylene promotes fruit ripening, senescence, and responses to pathogens and abiotic stresses. Its biosynthesis is regulated by developmental processes as well as by numerous external stresses. To understand the role of ethylene in plant functions, it is important to know how this gaseous hormone is synthesized, how its production is regulated, and how the signal is transduced. Morphological changes in dark grown (etiolated) seedlings treated with ethylene or its metabolic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), have been termed the “triple response”. The exaggerated curvature of the apical hook (1), radial swelling of the hypocotyl (2), and shortening of the hypocotyl and the root (3) are the unmistakable features of this ethylene response. ACC synthase catalyzes the committed step in ethylene biosynthesis, the conversion of S-adenosyl-methionine to ACC (Capitani et al., 1999) and requires pyridoxal 5’-phosphate (PLP) as a cofactor for activity. Over the past decade, the triple response phenotype has been exploited to screen for mutants that are defective in ethylene responses (Guzman and Ecker, 1990; Bleecker and Kende, 2000). Etiolated *Arabidopsis* seedlings with minor or no phenotypic response upon ethylene application are termed ethylene-insensitive (*ein*) or ethylene-resistant (*etr*) mutants.
A second plant hormone, auxin (indole-3-acetic acid, IAA) regulates cell division and expansion, vascular differentiation, lateral root development, and apical dominance. Despite intensive studies for over more than fifty years, the current understanding of auxin biosynthesis remains incomplete (Cohen et al., 2003; Pollmann et al., 2006). Although many different pathways of auxin synthesis have been proposed in the past (Woodward and Bartel, 2005), no auxin-deficient Arabidopsis mutant has been identified to date, suggesting that either the mutants are lethal or, alternatively, that redundant biosynthetic pathways exist (Pollmann et al., 2006). Furthermore, plants are believed to use, depending on their developmental stage, several different substrates, either tryptophan or its precursors such as indole or indole-3-glycerophosphate, respectively, as starting points for the synthesis of IAA. However, no enzyme of a tryptophan-independent pathway has been identified so far. The notion of tryptophan-independent pathways for IAA biosynthesis came from the observation that tryptophan synthase-deficient mutants of A. thaliana had higher than normal levels of IAA conjugates (Normanly et al., 1993). However, it was subsequently shown that the unstable tryptophan precursor indole-3-glycerol phosphate, which accumulates in the mutant, chemically decomposes to produce IAA and thus the notion of a tryptophan independent IAA biosynthesis was abandoned (Müller and Weiler, 2000). Therefore, so far, the only definitive route of IAA biosynthesis is from tryptophan and is thought to proceed via indole-3-pyruvic acid and indole-3-acetaldehyde. In this pathway, tryptophan aminotransferase, which converts L-tryptophan to indole-3-pyruvic acid, and indole-3-pyruvic acid decarboxylase, which catalyzes the formation of indole-3-acetaldehyde, require PLP for their enzymatic activities. Hence, enzymes involved in auxin biosynthesis are also dependent on PLP as a cofactor.

Briefly, the functions of the other classical hormones can be defined as follows: Cytokinins are adenine derivatives first identified by their ability to promote cytokinesis (Kakimoto, 2003). Jasmonic acid (JA), and more so its methyl ester are volatile signals that modulate pollen development and responses to pathogen infection (Turner et al., 2002). The brassinosteroids (BR) regulate cell expansion and photomorphogenesis (light regulated development) (Wang and He, 2004). Gibberellins are diterpenoid compounds that promote germination, stem elongation, and the induction of flowering (Gomi and Matsuoka, 2003). The sesquiterpenoid abscisic acid (ABA) promotes seed dormancy and is involved in several stress signalling pathways (Himmelbach et al., 2003; Gray, 2004).

It has been a conundrum how such a small number of chemically simple molecules could account for the observed diversity of cellular responses. Genetic screens have been very useful in identifying genes involved in hormone signal transduction. However, although these screens were originally designed to identify specific components involved in early hormone signaling, mutations in these genes often confer changes in sensitivity to more than one hormone at the
whole plant level (Gazzarrini and McCourt, 2003). For example, the auxin-resistant mutant \textit{axr1} also exhibits resistance to exogenous ethylene (Lincoln et al., 1990), and the ethylene-insensitive mutant \textit{eir2} (also known as \textit{pin2}, \textit{agr1} and \textit{wav6}) shows defective auxin responses and transport (Luschnig et al., 1998). In addition, part of the seedling growth response to ethylene is dependent on auxin biosynthesis as revealed by the study of the \textit{weak ethylene insensitive 2} and 7 mutants (Stepanova et al., 2005). Furthermore, hypocotyl elongation in the light involves gibberellins (Vriezen et al., 2004) and brassinosteroid hormones, along with cytokinin, in what appears to be a complex interaction between the three different hormone biosynthetic pathways (De Graauwe et al., 2005; Smets et al., 2005).

Taken together, these facts indicate that a linear representation of hormone signaling pathways controlling a specific aspect of plant growth and development is not sufficient; rather they interact with each other and with a variety of developmental and metabolic signals. From all of this, it has become clear that there is a significant amount of ‘cross-talk’ between the various hormone-dependent signalling pathways. We will use the described phenomena to understand the potential role of these hormones in the observed phenotypes of \textit{pdx1} plants. Even though the highly similar (87% identical), functional PDX1 proteins (PDX1.1 and PDX1.3) can synthesize PLP \textit{in vitro} at comparable rates (Tambasco-Studart et al., 2005; Tambasco-Studart et al., 2007), and the amino acids that are important for enzymatic activity in \textit{Bacillus subtilis} (D24, K81, K149) (Strohmeier et al., 2006) are conserved (Tambasco-Studart et al., 2007), their mutant phenotypes are drastically different from one another. \textit{pdx1.3} roots are much shorter than those of \textit{pdx1.1} or the WT and the rosette leaves of the \textit{pdx1.3} mutant seedlings exhibit a slightly chlorotic phenotype. This section is aimed at understanding the physiology of the observed phenotypic distinctions between the two \textit{pdx1} mutants. As a possible contributing factor behind these distinctions we will look into how these two proteins are hormonally regulated. Additional to the hormone signalling pathways, a second level of regulation resides in integration of signal transduction routes and is described below.

### 4.0.2 Regulation by \textit{cis} elements

Plant signal transduction pathways often converge at the promoter level to regulate the transcription of a common target. A \textit{cis}-regulatory element or \textit{cis}-element is a region of DNA which regulates the expression of genes located on that same strand. These \textit{cis}-regulatory elements are often binding sites of one or more \textit{trans}-acting factors. In this case, the physiological response is the result of complex interactions between the different signaling pathways.
A list of cis acting elements and their associated responses are as follows:

1) The Ethylene-Responsive Element (ERE) identified as the GCC box (AGCCGCC), is commonly found in the promoter region of ethylene-inducible defense genes (Shinshi et al., 1995). The ERE has also been shown to be involved in jasmonic acid signal transduction, pathogen attack and ozone stress (Koyama et al., 2003). ERE binding factors that interact with the GCC box have been demonstrated to be transcription factors, which respond to extracellular signals to modulate GCC box-mediated gene expression positively or negatively (Ohme-Takagi and Shinshi, 1995; Shinshi et al., 1995).

2) An auxin-response element (AuxRE) with the sequence TGTCTC, has commonly been identified in promoters of many auxin responsive genes (Ulmasov et al., 1997).

3) Potential binding sites for members of the MYB and MYC transcription factor families are involved in gene regulation in response to various stresses including drought, cold and abscisic acid (Abe et al., 2003).

4) The WRKY element has a DNA binding site involved in gene regulation under pathogen associated stress and in response to salicylic acid treatment (Yu et al., 2001).

5) The ABRE (ABA responsive element) element is associated with gene expression in response to osmotic stress (Iwasaki et al., 1995).

6) The C-repeat/DRE low temperature responsive element acts independent of abscisic acid (Baker et al., 1994).

7) TGA is an element which is involved in pathogen attack and salicylic acid treatment (Schindler et al., 1992).

Our prime interest resides in determining the potential factors behind the striking difference of the phenotype of the two pdx1 mutants. Initially an in silico search of the two PDX1 promoters for the presence of cis acting elements will give some hints about the regulation patterns. How these elements can contribute to the regulation of PDX1.1 and PDX1.3 expression will then be discussed. We will focus on the involvement of ethylene, but will also consider other hormones and their binding motifs, to ascertain if such hormones are also affected in a cross talk with the ethylene signal transduction pathway. An example illustrating this concept is the interactions between the ABA, ethylene and sugar signalling pathways. Growth on high concentrations of glucose or sucrose inhibits chloroplast development (To et al., 2002, 2003) and the breakdown of seed storage lipids (Martin et al., 2002; To et al., 2002). This inhibitory effect has been used to isolate sugar-hypersensitive (Nemeth et al., 1998) and resistant (Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000; Pego et al., 2000; Gibson et al., 2001) mutants of Arabidopsis. Characterization of sugar-hypersensitive and resistant mutants has provided evidence for interactions between sugar- and phytohormone-response pathways during very early seedling development. For example, the prl1 mutant of Arabidopsis, which is hypersensitive to the
inhibitory effects of high concentrations of exogenous sucrose and glucose on early seedling development, is also hypersensitive to cytokinin, ABA, ethylene, and auxin (Nemeth et al., 1998; Salchert et al., 1998). In addition, two Arabidopsis mutants that are resistant to the inhibitory effects of exogenous sucrose and glucose on early seedling development, sis1 (Gibson et al., 2001) and gin4 (Rolland et al., 2002), are allelic to the ethylene constitutive response mutant, ctr1 (Kieber et al., 1993). Similarly, the ethylene over producer mutant, eto1, (Guzman and Ecker, 1990) is resistant to the inhibitory effects of glucose and sucrose on early seedling development (Zhou et al., 1998; Gibson et al., 2001). Conversely, the ethylene insensitive mutants etr1-1 (Bleecker et al., 1988) and ein4b (Roman et al., 1995) are hypersensitive to sucrose- and glucose-mediated inhibition of early seedling development (Zhou et al., 1998; Gibson et al., 2001). In addition, exogenous ethylene allows wild-type Arabidopsis to grow and develop relatively normal shoot systems on otherwise inhibitory concentrations of exogenous glucose and sucrose (Zhou et al., 1998). These results indicate that an increased ethylene response or levels can result in increased resistance to sucrose and glucose and that a decreased ethylene response or level can lead to sucrose and glucose hypersensitivity during early seedling development. Therefore, the ability of the individual pdx1 knockouts to survive high sucrose concentrations will provide additional information on the ethylene responsiveness and/or rates of its biosynthesis.

However, it is important to note that the experimental data provided on the regulation of the PDX1’s in this section is only preliminary. Hence, in order to permit definitive conclusions on the regulation of the PDX1 proteins, several experiments need to be carefully repeated and the results verified by appropriate statistical treatment.

Results

4.0.1 Total vitamin B6 in pdx1.3 plants

Root growth of pdx1.3 seedlings is severely retarded in comparison with either the pdx1.1 mutant or wild type (WT) on medium lacking vitamin B6 (Chen and Xiong, 2005; Titiz et al., 2006). However, supplementation of the medium with 5 µM pyridoxine (PN) restores the growth of both of the mutants to that of WT. It has already been shown in chapter 3 that the total vitamin B6 level in the shoot tissue obtained from pdx1.3 plants is lower than in pdx1.1 and WT plants. In order to assess the role of vitamin B6 as a factor contributing to the short root phenotype, we also analysed the vitamin B6 level in root tissue of the pdx1 mutants and compared it to that of the shoot (Figure 1).
Figure 1. Vitamin B6 levels in root versus above ground tissue. The seedlings were grown in sterile medium lacking pyridoxine. Above ground organs and root samples at 2 weeks old were harvested, frozen in liquid nitrogen and stored at -80°C until analysis. The amount of vitamin B6 in the collected samples (n=3) was determined by the microbiological assay described in experimental procedures.

Generally, vitamin B6 levels in the shoots are higher on a fresh weight basis, than in the roots. But the level of vitamin B6 in pdx1.3 roots is nearly half the amount of the level found in pdx1.1 roots and one third of the amount found in WT. Yet, it is important to note that the ratio of the vitamin B6 level in shoot and root is the same in all the three genotypes.

The lengths of roots of pdx1.3 mutant seedlings after 6 days of growth were roughly proportional to the external pyridoxine supply, saturation was reached at circa 1 µM (Figure 2).
Figure 2. Rescue of *pdx1.3* root length by different concentrations of pyridoxine. The average root length of 30 seedlings in sterile culture, 6 days after germination is shown. Black bars: *pdx1.3*. Gray bar: WT.

### 4.0.2 Etiolated phenotype of *pdx1* seedlings

As mentioned earlier, ethylene is necessary for maintenance of the apical hook in *Arabidopsis* (Knee et al., 2000). As one of the enzymes involved in ethylene biosynthesis requires PLP as a cofactor, i.e. ACC synthase, the *pdx1* mutants were grown in the dark and their phenotype was analysed in the absence of vitamin B6 supplementation. Compared with the *pdx1.1* and WT seedlings, the *pdx1.3* mutant is impaired in its ability to form the apical hook curvature, while the apical hook curvature of the *pdx1.1* mutants is intermediate between that of the *pdx1.3* and the wild type. The phenotypes of both mutants reverted to normal (Figures 3 - 5) with addition of either ACC or pyridoxine (Figures 3 - 5).
Figure 3. Phenotype of *pdx1* and WT seedlings grown in continuous dark. Top row: in sterile medium without vitamin B6, middle row: in medium supplemented with 1 µM ACC and lower row: in medium supplemented with 5 µM PN. After vernalization, the etiolated seedlings were kept in complete darkness for 3 days at 22°C. The plants were taken out of the dark and their pictures immediately taken under the binocular microscope. The captured pictures of approximately 50 etiolated seedlings were analyzed and the angle of the apical hook curvature was measured, as indicated in the left picture.
Figure 4. Apical hook angle restoration with ACC. Black bars, WT; gray bars, pdx1.1; white bars pdx1.3. The seedlings were grown in media with and without the indicated amounts of ACC. The captured pictures of approximately 50 etiolated seedlings were analyzed. The error bars show the calculated standard deviation of the mean.

Figure 5. Apical hook angle restoration with 5 µM PN. The seedlings were grown in media with and without supplementation of 5 µM of pyridoxine. The captured pictures of approximately 50 etiolated seedlings were analyzed. The error bars show the calculated standard deviation of the mean.
4.0.3 Ethylene production in *pdx1* plants

Plants can detect and respond to invasion by microorganisms (Somssich et al., 1986). Bacterial flagellin, the protein subunit of the bacterial flagellum, is recognised in several plant species by a highly sensitive and specific perception system (Felix et al., 1999). The most conserved partial sequence of flagellin, from *Xanthomonas axonopodis citri*, consisting of 22 N-terminal amino acids (flg22) is sufficient to elicit the full response in a number of plant species, including tomato and *Arabidopsis thaliana* (Gomez-Gomez et al., 1999). The peptide flg22 induces defense-related responses, such as an oxidative burst, ethylene production, callose deposition and medium alkalization in cell suspension cultures, in addition to activation of genes coding for pathogenesis related proteins (Felix et al., 1999; Gomez-Gomez et al., 1999). Changes in the sequence of synthetic flagellin peptides resulted in a reduction of elicitor activity or antagonistic inhibition of the response, demonstrating the specificity of this perception system (Felix et al., 1999). As ethylene production is a characteristic of this response, it can be used as a method to assess the rate of production in WT plants versus mutants. Thus, the rate of ethylene production in *pdx1* mutant seedlings compared to WT was monitored in the absence and presence of vitamin B6 supplementation. In the absence of vitamin B6 supplementtion, *pdx1.3* mutants indeed produce lower amounts of ethylene compared to *pdx1.1* or WT (Figure 6). The response of *pdx1.1* is intermediate between that of *pdx1.3* or the WT. Growth of plants in the presence of pyridoxine results in the restoration of ethylene production to WT levels in both *pdx1* mutants (Figure 6).

![Figure 6](image-url)  
*Figure 6.* Ethylene production after flg22 elicitor stimulation; the black bars indicate ethylene production from the indicated genotype seedlings after 6 hours and the white bars are the respective controls, without any elicitor added (n=15). The error bars show the standard deviation of the mean.
4.0.4 Sucrose sensitivity of \(pdx1.1/PDX1.1\) \(pdx1.3/pdx1.3\) seedlings

Reduced ethylene production is known to result in sucrose hypersensitivity at the seedling stage, which is characterized by a lack of the ability to form chlorophyll (Gibson et al., 2001). To investigate a secondary effect of the reduced ethylene biosynthesis, a segregating population of \(pdx1.1/PDX1.1\) \(pdx1.3/pdx1.3\) seedlings were analyzed for their ability to grow in the presence of sucrose. Single copy \(PDX1.1\) plants are chlorotic and have 5% of the total vitamin B6 content of WT, i.e. the lowest content of all viable seedlings analysed (chapter 3). A feature which can be used to identify them within the population. A segregating population of \(pdx1.1/PDX1.1\) \(pdx1.3/pdx1.3\) seedlings grown on medium containing 1.5% sucrose had an even more bleached phenotype than normally observed, and growth was arrested at the seedling stage. Seedlings grown in medium without sucrose (Figure 7) showed the ability to form chlorophyll. The single copy \(PDX1.3\) plants, grown under the same conditions did not show any bleaching, nor did the WT (results not shown). The sucrose hypersensitivity is most apparent in the top right picture in Figure 7, in the areas of the seedling that are touching the sucrose containing medium.
Figure 7. *pdx1.1/PDX1.1 pdx1.3/pdx1.3* seedlings with and without sucrose. For sucrose sensitivity tests, the mutant plants were grown in vitamin B6 free medium with and without 1.5% sucrose for 12 days. The pictures in the same row indicate two examples. In the top left picture the medium with sucrose is touching the whole seedling, on the top right only the area of the seedling touching the medium shows a bleached phenotype.

### 4.0.5 Distinct binding motifs in the *PDX1.1* and *PDX1.3* promoters

Analysis of the PDX1 promoter regions allowed us to identify a putative ethylene responsive element, in the region upstream of the *PDX1.3* coding sequence, whereas this element is absent from the 1400 bp region upstream of the *PDX1.1* gene (Table 1 and Figure 8). In addition, the TGTCTC sequence of the auxin response element could be identified at – 859 bp upstream of the translation start site of *PDX1.3*. Like the ethylene responsive element, this auxin responsive element is not present in the *PDX1.1* promoter region (Table 1 and Figure 8). Furthermore, also as described by Denslow (2005) both gene promoters contain potential binding sites for members of the WRKY, MYB and MYC transcription factor families. Other stress-related regulatory motifs, including the abscisic acid responsive element (ABRE), C repeat/drought responsive element (C repeat/DRE) and TGA binding sites, were found in one or the other, but not in both of the functional *PDX1* genes.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>PDX1.1</th>
<th>PDX1.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Locus</strong></td>
<td>At2g38230</td>
<td>At5g01410</td>
</tr>
<tr>
<td>cold</td>
<td>MYB (4, WAACCA, CNGTTR)</td>
<td>C/DRE (1, CCGAC)</td>
</tr>
<tr>
<td></td>
<td>MYC (3, CANNTG)</td>
<td>MYB (2, CNGTTR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MYC (4, CANNTG)</td>
</tr>
<tr>
<td>osmotic stress</td>
<td>ABRE (3, CACGT)</td>
<td>C/DRE (1, CCGAC)</td>
</tr>
<tr>
<td>ozone</td>
<td></td>
<td>ERE (1, AGCCGCCC)</td>
</tr>
<tr>
<td>pathogen stress</td>
<td>TGA (1, TGACGTGG)</td>
<td>ERE (1, AGCCGCCC)</td>
</tr>
<tr>
<td></td>
<td>WRKY (5, TTGAC)</td>
<td>WRKY (4, TTGAC)</td>
</tr>
<tr>
<td>ABA</td>
<td>MYB (4, WAACCA, CNGTTR)</td>
<td>MYB (2, CNGTTR)</td>
</tr>
<tr>
<td></td>
<td>ABRE (3, CACGT)</td>
<td>MYC (4, CANNTG)</td>
</tr>
<tr>
<td>ethylene</td>
<td></td>
<td>ERE (1, AGCCGCCC)</td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td></td>
<td>ERE (1, AGCCGCCC)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>TGA (1, TGACGTGG)</td>
<td>WRKY (4, TTGAC)</td>
</tr>
<tr>
<td></td>
<td>WRKY (5, TTGAC)</td>
<td></td>
</tr>
<tr>
<td>Auxin</td>
<td></td>
<td>AuxRE (1, TGTCT)</td>
</tr>
</tbody>
</table>

Table 1. Potential binding sites for stress-responsive regulatory elements. Motifs identified in the 1.4 kb upstream regions of *PDX1.1* and *PDX1.3* before the *in silico* identified translation start codon are shown. The signature of each motif and the number of copies present are indicated. (N:A/T/G/C, R: A/G, W: A/T) taken from Denslow (2005).
**Figure 8.** Binding sites for stress-responsive regulatory elements. Motifs identified in the 1.4 kb promoter regions of PDX1.1 and PDX1.3 upstream of the in silico identified translation start codon are shown.
4.0.6 Regulation of \textit{pdx1} genes during biotic stress

Salicylic acid (SA), jasmonic acid (JA) and ethylene have all been implicated in pathogen stress responses (Kunkel and Brooks, 2002). In order to determine the regulatory role (if any) of SA application on the \textit{PDX1} genes, we determined the change in \textit{PDX1.1} and \textit{PDX1.3} transcript abundance during 48 hours after the application of SA to the rosette leaves of soil grown WT plants. Six hours after application of 1 mM of SA, a sudden transient increase in the \textit{PDX1.1}, but not in the \textit{PDX1.3} transcript was observed (Figure 9).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Transcript abundance of \textit{PDX1.1} and \textit{PDX1.3} in \textit{Arabidopsis} rosette leaves after salicylic acid and methyl jasmonate treatment. Adaxial and abaxial surfaces of rosette leaves from 4 week-old, soil grown plants (WT Col-0) were sprayed with either 0.5 mM methyl jasmonate or 1 mM salicylic acid, lower and upper panels respectively. After drying for 1 hour, plants were covered with a plastic bag to maintain humidity. Leaf tissue was collected from three plants at 0, 6, 12, 24 and 48 hours. The results shown are the mean and standard deviation of three different plant samples. Samples of plant material collected were immediately frozen in liquid nitrogen and kept at -80°C. The transcript level relative to ‘time 0’ was measured by quantitative RT-PCR.}
\end{figure}

In order to investigate the role of the so called GCC box, the change in \textit{PDX1.1} and \textit{PDX1.3} transcript abundance after the application of MeJA to the rosette leaves of WT plants and after incubation in an ethylene (10 ppm) filled chamber was analyzed. As shown in Figure 9, the application of 0.5 mM MeJA leads to a transient rise in the \textit{PDX1.3} but not in the \textit{PDX1.1}
transcript. In response to ethylene, the \textit{PDX1.3} transcript abundance increases 3-fold relative to the control, whereas \textit{PDX1.1} transcript abundance does not change significantly (Figure 10).

![Figure 10](image-url)

\textbf{Figure 10.} Transcript abundance of \textit{PDX1.1} and \textit{PDX1.3} in \textit{Arabidopsis} rosette leaves 12 hours after exposure to ethylene (10 ppm). The results shown are the mean and standard deviation of three different experiments.

\textbf{4.0.7 Analysis of \textit{A. thaliana} with T-DNA insertions in the putative AuxRE and ERE of the \textit{PDX1.3} promoter region}

The diagram in Figure 11 (a) indicates the positions of T-DNA insertions in the putative ethylene and auxin response elements of the 1.4kb upstream region of \textit{PDX1.3}, i.e. SALK\_113583 and SALK\_057214, respectively. Figure 11 (b) shows a PCR analysis of identified homozygous lines of the mentioned insertions.
Root growth of the homozygous lines was examined in comparison with *pdx1.3* and WT, in the absence and presence of vitamin B6 (Figure 12). Roots of plants homozygous for T-DNA insertions in either the ERE or AuxRE elements grow at a slower rate than the WT, but better than the *pdx1.3* plants, respectively. In both cases, the growth defect could be alleviated by addition of 5 µM pyridoxine to the growth medium (Figures 12 and 13).

**Figure 11.** (a) Diagram showing the genomic localization of the putative ethylene (red box) and auxin (green box) response elements and their T-DNA insertions, SALK_113583 and SALK_057214, respectively. (b) Identification of SALK_113583 and SALK_057214 lines homozygous for a T-DNA insertion by PCR. The first panel shows results of PCR reactions that were designed to detect the T-DNA insertions in SALK_113583 and SALK_057214 lines (the LB and FW primers were used). The second panel shows the WT promoter PCR amplification (FW and BW primers were used) of the same plants and the WT plant as a control.
Figure 12. Partial rescue by vitamin B6 application of the short root phenotype of the putative ERE and AuxRE T-DNA insertion lines, SALK_113583 and SALK_057214, respectively. The seedlings homozygous for the T-DNA insertions were grown for 10 days whereas the WT seedlings were grown for 9 days.
**Figure 13.** Partial rescue of the short root phenotype of the putative ERE and AuxRE T-DNA insertion lines SALK_113583 and SALK_057214, respectively, compared with *pdx1.3* seedlings. The seedlings were grown for 10 days and their root length measured.

### 4.0.8 Inhibition of root growth by BA and NAA

A literature survey was performed to search for characterized genes with a short root phenotype similar to *pdx1.3*. The survey revealed that plants carrying mutations in the *POLARIS* (*PLS*) gene of *A. thaliana* (Casson et al., 2002) have a similar short root phenotype. In addition, *pls* roots are hypersensitive to exogenous cytokinins and less sensitive to growth inhibitory effects of auxin (Casson et al., 2002). Thus, it was concluded that the *PLS* gene is required in auxin-cytokinin homeostasis and is necessary to modulate root growth and vascular patterning (Casson et al., 2002). When we tested the *pdx1.3* roots for sensitivity to exogenously applied cytokinin and auxin, we did not find any significant difference in the response pattern in comparison to WT plants (Figures 14 and 15, respectively).
Figure 14. Inhibition of root growth by BA (benzyladenine). Seedlings were grown for 7 days in the presence of vitamin B6 (5 µM PN) on plates with BA; White bars: -BA, gray bars: 10 nM BA and black bars: 1 µM BA, and their respective root growth was measured. The average elongation rate and the standard deviation of the mean of 20 seedlings are shown in relation to control without BA (100%).

Figure 15. Inhibition of root growth by 1-naphthalene acetic acid (NAA). White bars, WT; gray bars, pdx1.1 and black bars, pdx1.3 seedlings were grown for 4 days in the presence of vitamin B6 (5 µM PN), then transferred to plates with the indicated concentrations of NAA and root growth was measured after 3 days. The average elongation rate and the standard deviation of the mean of 20 seedlings are shown in relation to the control plates without NAA.
**Discussion**

In chapter 3, phenotypes of *pdx1* mutant plants were described. In this chapter, we have additionally discovered that the vitamin B6 content of the roots of *pdx1.3* plants is much less than that of either *pdx1.1* or WT. However, the ratio of vitamin B6 in the shoot versus the root remains the same in all the samples analyzed. Even though the vitamin B6 content of the *pdx1.3* shoots was reduced by the same factor as in roots, the mutant phenotype of the root is much more exaggerated than that of the shoot. A threshold mechanism of vitamin B6 biosynthesis could be postulated as a contributing factor for the observed difference. Such that the absolute amount of vitamin B6 in the shoot still remains more than the absolute amount measured in the root, and that the lack of vitamin B6 availability under a certain threshold leads to a stunted root phenotype.

The modulation of apical hook formation in *pdx1* mutants indicates that either ethylene perception or ethylene biosynthesis is impaired. The restoration of the apical hook angle to that of WT by supplementation with ACC and pyridoxine (Figures 3-5) indicates that a lack of ethylene perception is not the reason behind the etiolated phenotype, but instead is due to a lack of ethylene biosynthesis. Indeed, ethylene biosynthesis measurements of the two mutants indicate that *pdx1.3* is much more retarded in this ability compared to *pdx1.1* or WT. This may be an indirect consequence due to a shortage in the PLP cofactor required for the enzymatic activity of ACC synthase. Additionally, a reduction in ethylene biosynthesis may indirectly lead to reduction of auxin biosynthesis at the tip of the primary root (Stepanova et al., 2005). The indirect role of ethylene in root growth became apparent recently by the action of the genes *WEI2* and *WEI7*, which encode the α- and β-subunit, respectively, of a rate-limiting enzyme of tryptophan biosynthesis, anthranilate synthase (Stepanova et al., 2005). *WEI2* and *WEI7* are also known as *ASA1* and *ASB1*, respectively. *wei2* and *wei7* mutant plants were found to possess root-specific ethylene insensitivity. It was shown in the same study that upregulation of *WEI2/ASA1* and *WEI7/ASB1* by ethylene results in the accumulation of auxin in the tip of the primary root, whereas loss-of-function mutations in these genes prevent the ethylene-mediated auxin increase (Stepanova et al., 2005). As the tryptophan-dependent route is the only validated route of IAA biosynthesis (Müller and Weiler, 2000) which requires PLP-dependent enzymes, the short root phenotype of the *pdx1.3* mutant can be attributed to a lack of PLP that is used in the biosynthesis of both IAA and ethylene.

As a secondary effect of lack of ethylene biosynthesis, single copy *PDX1.1* seedlings were shown to be highly sensitive to exogenous application of sucrose. Previously, it has been shown that during early seedling development, the phytohormone signaling pathways interact and that decreased ethylene levels can lead to sucrose hypersensitivity during early seedling
development (Gazzarrini and McCourt, 2003). Thus, the sucrose sensitivity of single copy PDX1.1 can be attributed to a reduction in ethylene biosynthesis. This phenomenon needs to be investigated in future experiments with the individual pdx1 mutants, in particular with pdx1.3.

As a second approach to explore the underlying mechanism behind the observed pdx1 phenotypes, we analyzed how the two PDX1 genes might be regulated at the promoter level. A number of studies have linked vitamin B6 to stress responses in various organisms in which the expression of the PDX1 genes have been affected by several stress conditions. For example, in the bacterium Bacillus subtilis, expression of the PDX1 homolog (YaaD) is enhanced after treatment with the superoxide generator, paraquat (Antelmann et al., 1997); Saccharomyces cerevisiae, increases its transcript accumulation of the functional PDX1 homolog, SNZ1, at entry into stationary phase, a time of high oxidative stress (Braun et al., 1996; Padilla et al., 1998). In plants, the expression of the PDX1 genes and some of the vitamin B6 salvage pathway genes (such as pyridoxal kinase, PdxK) were also shown to be upregulated during conditions leading to oxidative stress. In Arabidopsis thaliana, UV-B radiation causes an increase in the transcript level of PDX1.3 (Brosche et al., 2002). In addition, PdxK in A. thaliana has been implicated in salt tolerance and shows increased transcript levels during cold stress (Shi et al., 2002). In Phaseolus vulgaris, abscisic acid and wounding induce an increase in the PDX1 transcript level (Graham et al., 2004). Furthermore, application of salicylic acid and ethylene, chemical mediators of plant-pathogen defense responses, a process involving high oxidative stress, resulted in increases in the PDX1 transcript abundance of Hevea brasiliensis (rubber tree) (Sivasubramaniam et al., 1995). Even though past research in this area already indicates that the PDX1 genes are regulated by conditions leading to oxidative stress and other abiotic stress, not much is known about the differential regulation patterns (if any) of the two functional A. thaliana PDX1 genes (PDX1.1 and PDX1.3), under the same stress conditions. The PDX1.3 promoter, unlike the PDX1.1 promoter, has an ERE as well as an AuxRE binding site (Table 1 and Figure 8). Exposure of Arabidopsis to ethylene resulted in an increase in the PDX1.3 transcript but not in that of PDX1.1. These results are in accordance with the presence of the putative ERE element in the promoter of PDX1.3, but not of PDX1.1. It is important to note however, that the ERE binding element can be activated in ways which do not involve ethylene (Yamamoto et al., 1999). ERE, despite what the name implies, is not just an ethylene activated transcription factor but has also been implicated in responses to pathogen attack (Gutterson and Reuber, 2004), ozone stress (Grimmig et al., 2003) and jasmonic acid. Indeed, MeJA treatment causes a three-fold increase within six hours of the PDX1.3 transcript level which drops sharply thereafter, whereas the PDX1.1 transcript level does not significantly change at all times analyzed. These preliminary results indicate that even though the enzymatic function of the two highly similar PDX1 proteins are the same, the regulation pattern of their expression is
remarkably different. In an attempt to take this argument one step further and examine the hypothesis that the phenotypical differences are a result of the differential regulation pattern, putative T-DNA insertion lines of the respective ERE and auxin response elements in the PDX1.3 promoter were analyzed. The root phenotype of the T-DNA insertion lines was not as pronounced as that of the pdx1.3 insertion lines, but exogenous pyridoxine application partially restored the root growth to normal. The short root phenotype of pdx1.3 can therefore also partially be attributed to the malfunctioning of the, normally to be activated, transcription factor binding site which at some point may involve activation of ERE or AuxRE by ethylene or auxin, respectively. Future work to establish the role of the putative ERE or Aux RE requires systematic deletion of these elements and a full expression analysis.

The TGA elements, which are only present in the PDX1.1 promoter, are associated with gene expression during pathogen attack and salicylic acid treatment (Durrant and Dong, 2004). The transient rise in PDX1.1 but not in PDX1.3 transcript abundance after the SA treatment and the transient rise in the PDX1.3 but not in PDX1.1 transcript after the MeJA treatment can be explained by the antagonistic nature of the SA and JA signaling pathways during pathogen attack (Kunkel and Brooks, 2002; Pieterse and Van Loon, 2004) as has already been postulated by Denslow et al. (2005). The mutually exclusive induction of the PDX1 genes by SA and MeJA treatments is likely due to the unique presence of the corresponding transcription factor binding elements in their respective promoters. As the antioxidant property of pyridoxine is well documented (Benderitter et al., 1996; Ehrenshaft et al., 1999; Tadera et al., 2003; Chumnantana et al., 2005; Denslow et al., 2005), a likely explanation of the initial rise and then a drop in the PDX1 transcripts in both SA and MeJA treatments is that the plant initially tries to cope with the SA or MeJA mimicked pathogen attack by increasing its antioxidant level, but as the treatment continues, it decides to control the pathogen locally, i.e. where the pathogen signal is active, by programmed cell death, which is characteristic of the Hypersensitive Response (HR) (Goodman, 1994). This may indicate a regulation pattern which complies by decreasing antioxidants in tissues mounting a so called pathogen response. In many cases, the induction of defense responses is accompanied by localized cell death at the site of pathogen entry (Murphy et al., 1999; Mishina and Zeier, 2007), which is often able to restrict the spread of the pathogen to cells within and immediately surrounding the lesions.

The two PDX1 promoters also varied in the presence of some abiotic stress response elements, such as C repeat/drought responsive element (C repeat/DRE) which is found in the PDX1.3 promoter and which promotes gene expression in response to high osmolarity, drought and cold, but its function is independent of abscisic acid (Yamaguchi-Shinozaki and Shinozaki, 1994; Pastori and Foyer, 2002). Whereas the abscisic acid responsive element (ABRE) which is
also associated with gene expression in response to osmotic stress and drought (Shinozaki et al., 2003), is only found in the PDX1.1 promoter.

As a third approach to understand the factors behind the observed pdx1 differential phenotypes we searched the literature to identify mutants with similar phenotypes. A survey of the literature revealed that the glutathione (GSH)-deficient mutant, root meristemless1 (rml1) (Vernoux et al., 2000) and the vitamin C-deficient mutant vitamin C1 (vtc1) (Lukowitz et al., 2001) have stunted root phenotypes. Yet, preliminary experiments indicated that supplementation with GSH or vitamin C failed to restore the growth of pdx1 roots (results not shown). Plants carrying mutations in the POLARIS (PLS) gene of A. thaliana (Casson et al., 2002) have short root phenotypes, and in addition have a defect in the vascular patterning of the leaves and typically produce more anchor roots at the root-hypocotyl junction, which is very similar to the pdx1.3 phenotype (Titiz, unpublished results). Microscopic analysis of the pls roots indicated that meristem cells divide less frequently than WT cells (Casson et al., 2002), which is again similar to the pdx1.3 roots (Chen and Xiong, 2005). The promoter of the PLS gene carries a copy of the putative AuxRE cis element (Casson et al., 2002), and expression of the PLS gene which encodes for a small polypeptide predicted to be required for correct leaf and vascular patterning is induced by auxin. In addition, pls roots are hypersensitive to exogenous cytokinins and less sensitive to growth inhibitory effects of auxin (Casson et al., 2002). Thus, it was concluded that the PLS gene is required in auxin-cytokinin homeostasis and is necessary to modulate root growth and vascular patterning (Casson et al., 2002). When we tested the pdx1.3 roots for sensitivity to exogenously applied cytokinin and auxin, we did not find any significant difference in the response pattern in comparison to WT plants. Another mutant reported in the literature with a stunted root phenotype is bls1 (brassinosteroids, light and sugar) mutant. It displays altered brassinosteroid response and sugar sensitivity. In the light, the bls1 mutant is dwarf and develops a short root, compact rosette, and exhibits delayed bolting (Laxmi et al., 2004). The pdx1.1/PDX1.1 pdx1.3/pdx1.3 mutants have also been reported to exhibit delayed bolting (chapter 3). The bls phenotype was partially rescued by the exogenous application of epibrassinolide (EBR). In order to test whether a defect in BR could be a contributing factor to the phenotype of the pdx1.3 mutant, we treated these plants with EBR, but did not observe restoration of root growth (results not shown).

**Conclusions**

pdx1.3 is reduced in its vitamin B6 content. This may inevitably lead to a reduced availability of PLP as a cofactor in certain metabolic reactions. In this context, PLP is required as a cofactor for ethylene and auxin biosynthesis, two hormones which are known to play a role in
root development. In an attempt to define the short root phenotype of the \textit{pdx1.3}, we determined the rate of ethylene biosynthesis, and discovered it to be less than that synthesized by \textit{pdx1.1} or WT. Due to the regulation of auxin production by ethylene (Stepanova et al., 2005), this may indirectly lead to a shortage in auxin biosynthesis, which might explain the impaired root growth of the \textit{pdx1.3} mutant. In addition to the shortage of the cofactor for the biosynthesis of two hormones which are required for root growth, a potential involvement of the putative AuxRE and ERE elements cannot be ruled out.

We can anticipate that distinct regulatory mechanisms for the expression of the two \textit{PDX1} genes might be related to promoter-specific \textit{cis} binding elements. As ethylene, SA and MeJA have been shown to stimulate the production of reactive oxygen species (Orozco-Cardenas and Ryan, 1999; Kawano and Muto, 2000; Yoshioka et al., 2001; Yoshioka et al., 2003), we can further speculate that the relative changes in the transcript abundance of the respective genes might be a possible mechanism to maintain and monitor the correct place, timing and dosage of antioxidant supply. Nevertheless, the insertional mutagenesis alone is not adequate to identify the functions of the respective promoter elements. Systematic deletion and expression analysis, e.g. stable expression of GUS fusions in \textit{planta} will be required.

**Experimental Procedures**

4.0.1 Plant material and growth conditions

\textit{Arabidopsis thaliana} (WT, ecotype Col-0) plants were grown in soil under a 16-h day (100 \textmu mol m\textsuperscript{-2} sec\textsuperscript{-1}) and 8-h night cycle at 22°C/19°C, respectively. When grown in sterile culture, unless stated otherwise, \textit{Arabidopsis} seeds were first surface-sterilized, plated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1.5% sucrose and 0.9% w/v agar and kept for 4 days in the dark at 4°C. Thereafter, the plates were transferred to continuous light (100 \textmu mol m\textsuperscript{-2} sec\textsuperscript{-1}) at 22°C. For recording the etiolated phenotype, the plants were kept in the dark for 3 days at 22°C, then taken out of the dark and their pictures taken immediately under the binocular microscope. The captured pictures of approximately 50 etiolated seedlings were analyzed and the angle of the apical hook curvature was measured. For the analysis of T-DNA insertions in the putative AuxRE and ERE motifs, the SALK\_057214 and SALK\_113583 lines, respectively, were analyzed after growing the mutant seedlings on vertical plates for 10 days with and without 5 \textmu M pyridoxine. For sucrose sensitivity tests, the double mutants, \textit{pdx1.1/PDX1.1 \textit{pdx1.3/pdx1.3}} seedlings, were grown in vitamin B6 free medium with and without 1.5% (w/v) sucrose for 12 days. For all hormone treatments, 4 week-old soil grown
plants were used. In all cases, samples of collected plant material were immediately frozen in liquid nitrogen and kept at -80°C until further use.

4.0.2 Hormone treatments

For salicylic acid treatment, adaxial and abaxial surfaces of *Arabidopsis* rosette leaves were sprayed with 1 mM salicylic acid (Sigma-Aldrich, Dreieich, Germany). Leaf tissue from three separate plants was collected at 0, 2, 6, 24, and 48 hours. For methyl jasmonate treatment, adaxial and abaxial surfaces of *Arabidopsis* rosette leaves were sprayed with 0.5 mM methyl jasmonate (Sigma-Aldrich, Dreieich, Germany). After drying for 1 hour, plants were covered with a plastic bag to maintain humidity. Leaf tissue was collected from three separate plants at 0, 2, 6, 24 and 48 hours. For ethylene treatments, plants were placed in a chamber filled with 10 ppm ethylene for 12 hours, while control plants were maintained in ethylene free conditions in another chamber under the same growth conditions for the same time. Rosette leaves of three separate plants were used. NAA (Casson et al., 2002) and EBR (Laxmi et al., 2004) treatments were done exactly as in the relevant publications indicated.

4.0.3 Isolation of RNA and quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen AG, Switzerland) and treated with RNase-free DNase (Qiagen) to remove traces of DNA, according to the manufacturer’s instructions. First strand cDNA synthesis was performed at 42°C for 60 min in a volume of 20 µl, containing 2 µg of total RNA, 20 pmol of oligo(dT) and 200 units of reverse transcriptase from the Advantage RT-for-PCR kit (Takara Bio Europe/Clontech, Switzerland). For transcript quantification, the equivalent of 50 ng of total RNA was employed. Quantification analyses were performed using an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA), by fluorescence-based real-time PCR with the fluorescent dye SYBR Green. The PCR conditions for all genes analysed were as follows: an initial denaturation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Relative mRNA abundance was calculated using the comparative ΔCt method and normalized against the constitutively expressed actin-2 (At3g18780) gene. The primers used for this quantification were: *PDX1.1* forward, 5’GTGAGGAGTGTGAACGGAGC-3’; *PDX1.1* reverse, 5’ GCACAACCAAATCATACGGC-3’; *PDX1.3* forward, 5’-ATAATTTCCGGATCCCGTTC-3’; *PDX1.3* reverse, 5’CATCATCATCCATGTTCGC-3’; Actin-2 forward, 5’-ATTCTTGCTTCCCTCAGCAC-3’; Actin-2 reverse, 5’-CCCCAGCTTTTTTAAGCCTTT-3’
4.0.4 Vitamin B6 bioassay

Samples for vitamin B6 analysis were obtained by growing seedlings in sterile medium in the absence of vitamin B6. Above ground organs and root samples were harvested at 2 weeks, frozen in liquid nitrogen and stored at -80°C until analysis. The amount of vitamin B6 in the collected samples was determined by employing the microbiological assay as described in Tambasco-Studart et al. (2005). The vitamin was extracted from the plant material (2–5 mg) by homogenization with a mortar and pestle in 0.02 M H$_2$SO$_4$, after which the sample was autoclaved at 121°C for at least 1 h. After extraction, the pH of the solution was adjusted to 5.2 using 3 M sodium acetate and centrifuged for 15 min at 10,000g. Levels of vitamin B6 in the supernatant were quantified by extrapolation to a standard curve obtained from growth of the *Saccharomyces carlsbergensis* strain ATCC 9080 in the presence of pyridoxine (Tambasco-Studart et al., 2005). All samples and standards were determined in triplicate.

4.0.5 Ethylene measurements

The Flg22 peptide was obtained from Peptron (Daejeon, South Korea). Prior to use, the peptide was dissolved in water (stock solutions of 1 to 10 mM) and diluted to a final concentration of 10 µM in a solution containing 0.1% BSA and 0.1 M NaCl. For assaying ethylene production, leaf slices were cut from plants grown for 10 days in sterile culture with and without 5 µM of pyridoxine. For sampling, five separate seedlings were collected (totaling to ~20 mg of fresh weight per assay) and transferred to 6-mL glass tubes containing 1 mL of water and the elicitor preparation to be tested. The tubes were closed with rubber septa, and ethylene accumulating in the free air space was measured by gas chromatography after 4h incubation. The average of 15 different samples was determined.
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5 General Discussion

Vitamin B6 is well renowned in the medical field for being involved in more bodily functions than any other single nutrient. The vitamin B6 biosynthetic pathway had been classically studied only in *E. coli*. Between 1999 and 2001, a novel pathway different to that in *E. coli*, characterized by the *PDX1* and *PDX2* genes was discovered. Since then it has been shown that *PDX1* and *PDX2* genes are widely distributed and conserved amongst organisms that make the vitamin and, furthermore, the vitamin has been found to play a novel important role as an antioxidant. Another aspect that makes this biosynthesis pathway interesting relates to the rise of multiple resistance to antibiotics and an imminent need to find new anti-bacterials. A recent study (Becker et al., 2006) highlights the challenge of identifying new antibiotic drug targets. The criteria which allow a specific protein to be classified as a possible drug target, are its conserved structure amongst a broad range of micro-organisms and the absence of this protein from the host organism (Kohanski et al., 2007). The presence of the *de novo* biosynthetic pathway in micro-organisms and plants, but its absence from humans makes it an important antibacterial or herbicidal target. Indeed, intellectual property or patent applications have recently been filed in the respective area (US20050287169, 2005; Intellectual Property ID:T001246, 2007). T001246 is a licensable technology for which a provisional patent application has been filed and describes the development of novel broad spectrum antibacterial agents and vaccines targeting the novel vitamin B6 synthesis pathway identified in *Bacillus subtilis* and *Listeria monocytogenes*. US20050287169 is a US patent application which contains methods and use of genes of pyridoxal 5'-phosphate (PLP) biosynthesis in *Bacillus subtilis* avirulent strains for vaccines, and methods for identification of antibacterial agents. In order to fulfill this goal, there is a need to fully understand and unravel the underlying mechanism behind the synthesis of vitamin B6 in microorganisms as well as plants. The purpose of this chapter is to give a comprehensive overview of vitamin B6 biosynthesis with a focus on plants and to draw conclusions on the topics addressed in the previous chapters by also taking into account the recent discoveries made in the same or related topics.

Vitamin B6 biosynthesis in plants

Recombinant expression, purification and biochemical characterization of the *A. thaliana* PDX1.1 and PDX1.3 proteins revealed that they are both able to catalyze the formation of PLP from either ribose 5-phosphate or ribulose 5-phosphate as the pentose phosphate sugar and either dihydroxyacetone phosphate or glyceraldehyde 3-phosphate (GAP) as the triose phosphate sugar, in the presence of ammonium sulfate as nitrogen donor. Interestingly, PDX1.3
appears to accept dihydroxyacetone phosphate more readily than PDX1.1. (Chapter 3). No activity of PDX1.2 was observed in any of the in vitro conditions tested, indicating that it cannot catalyze PLP biosynthesis. Moreover, complementation of the yeast vitamin B6 auxotrophic mutant snz1 with A. thaliana PDX1.1 and PDX1.3, but not PDX1.2, provided in vivo proof of the functional proteins in vitamin B6 biosynthesis. Others have also shown that PDX1.3 complements the auxotrophic yeast snz1 mutant (Chen and Xiong, 2005), but PDX1.1 was not included in this study. Surprisingly PDX1.3 was also found to complement the E. coli PNP synthase (pdxJ) mutant (Wagner et al., 2006). This finding is remarkable as PdxJ catalyzes a completely different reaction (Laber et al. 1999). However, as discussed by Raschle (2007), it is concluded that, even though very little sequence homology exists between the Pdx1 and PdxJ proteins of B. subtilis and E. coli, respectively, when examined in detail they display a considerable similarity both mechanistically and structurally. However, the nature of the substrates that Pdx1 would use to substitute for PdxJ remain to be defined, in particular the source of the pyridine nitrogen. Evidence for the functionality of PDX1 in vitamin B6 biosynthesis also came by means of a genetic approach, in which Wagner et al. (2006) identified a novel mutant of A. thaliana called rsr4-1 (for reduced sugar response), with aberrant root and leaf growth, that requires supplementation with vitamin B6 for normal development. Cloning of the mutated gene revealed that rsr4-1 carries a point mutation in the PDX1.3 gene, which results in a reduced vitamin B6 content. As Wagner et al. focused on the rsr4-1 mutation, they did not test if PDX1.1 complements the pdxJ mutant. While these results support our conclusion that PDX1.3 is functional in vitamin B6 synthesis, they remain short of testing the function of PDX1.1 in vitamin B6 biosynthesis.

On the other hand, sequence comparison studies predicted that PDX2 is a member of the class I glutaminase family, characterized by a Cys-His-Glu catalytic triad (Galperin and Koonin, 1997). Moreover, 15N-labelling studies in various eukaryotic and prokaryotic microorganisms harbouring the Pdx1 and Pdx2 genes led to the conclusion that the nitrogen atom in vitamin B6 is derived from the amide N of glutamine, rather than from glutamate as in E. coli (Tazuya et al., 1995; Tanaka et al., 2000), supporting the view that PDX2 is a glutaminase. In Chapter 2 and 3, the activity of A. thaliana PDX2 could not be demonstrated in vitro. The glutaminase activity of PDX2 has since been demonstrated in several organisms, i.e. Bacillus subtilis (Belitsky, 2004), Saccharomyces cerevisiae (Dong et al., 2004), Plasmodium falciparum (Wrenger et al., 2005; Gengenbacher et al., 2006) and, more recently, Arabidopsis thaliana (Tambasco-Studart et al., 2007). In each case, the activity of PDX2 was shown to be dependent on PDX1, and it was demonstrated that PDX1 and PDX2 function together as a glutamine amidotransferase. Glutamine amidotransferases are typically composed of two domains, i.e. a glutaminase and a synthase domain. In the glutaminase domain, glutamine is hydrolyzed to yield
glutamate and ammonia, the latter being then channelled to the synthase domain where it is utilized in the synthesis of the respective nitrogen-containing compound. This complex has now been shown to be in operation in most bacteria and plants (Raschle et al. 2005) and (Chapter 3). On the basis of substrate requirements (E. coli utilizes DXP as a substrate), the novel pathway characterized by the PDX1 and PDX2 genes is now termed the ‘DXP-independent’ pathway.

The localization of PLP synthase subunits

In plants, the formation of isopentenyl diphosphate and dimethylallyl diphosphate, the central intermediates in the biosynthesis of isoprenoids, is compartmentalized: the mevalonate (MVA) pathway is localized to the cytosol, in contrast, the more recently discovered MVA-independent pathway, which utilizes DXP, is localized to the plastids (Lichtenthaler, 1999; Eisenreich et al., 2001; Rodriguez-Concepcion and Boronat, 2002). Despite the compartmentalization of these two pathways, MVA-derived precursors can be used for the synthesis of isoprenoids in the plastid, and the non MVA-derived precursors can be exported to the cytosol in at least some plants, tissues, or developmental stages (Lichtenthaler, 1999; Eisenreich et al., 2001). An active uptake of IPP into isolated plastids was reported for several plants (Kreuz and Kleinig, 1984), while transport of IPP in the plastid-to-cytosol direction was shown recently to be mediated by a plastidial proton symport system (Bick and Lange, 2003). However, a literature search to date indicates that this exchange takes place only once the IPP has been made, and there is no indication that DXP or DXS (DXP synthase) might also occur in the cytosol. In E. coli, vitamin B6 is derived from DXP, and it was widely assumed that the pathway takes a similar route in plants and therefore would be localized to the plastid. In one preliminary study, it was actually claimed that DXP was used as a precursor of vitamin B6 in spinach chloroplasts (Julliard, 1992). Yet in another study, in Ginkgo biloba (Drewke and Leistner, 2001), label from DX or DXP was not incorporated into vitamin B6 in vivo. Therefore, since the discovery of the alternative pathway, the site of vitamin B6 de novo biosynthesis in plants had to be investigated. Via transiently expressed PDX1::GFP fusion proteins, we have shown that in A. thaliana all the PDX proteins localize to the cytosol (Tambasco-Studart et al., 2005). However, a recent publication has documented that the PDX1.3 protein localizes mostly to membranes. By generating stably transformed Arabidopsis plants expressing the GFP-PDX1.3 fusion protein, Chen and Xiong (2005), based on confocal microscopy analysis of epidermal leaves claimed that PDX1.3 associates mainly with the endomembrane system, including plasma membrane and the nuclear envelope, but also chloroplast outer membranes. However, a fraction of the protein was also localized in the cytosol (Figure 1 A-C). In root cells,
Figure 1. Localization of PDX proteins reported independently from this thesis. Localization of GFP-PDX1.3 fusion protein (A-F), by Chen and Xiong (2005). Guard cells of a fixed leaf epidermal peel of Arabidopsis seedlings (A-C). (A) GFP fluorescence, (B) red fluorescence of chlorophyll, (C) overlay of (A) and (B). Root cortical cells of fixed Arabidopsis seedling (D-F). (D) GFP fluorescence, (E) white-light image, (F) overlay of (D) and (E). Localization of PDX2-GFP fusion protein (G-I), by Denslow et al., (2007). (G, H, I) white-light image, GFP fluorescence, and DAPI staining, respectively, of a guard cell from a fixed epidermal peel expressing GFP-PDX2. Fluorescence is visible at the guard cell periphery and in the nuclei (arrows H, I).

The GFP signal was localized to the plasma membrane (Figure 1 D-F) (Chen and Xiong, 2005). Without the control showing the localization of GFP alone, it is difficult to comment if the GFP signal is exclusive to the membranes within the root cortical cell. On the other hand, Denslow et al. (2007) reported that in stably transformed Arabidopsis seedlings, a GFP-PDX2 fusion protein localized to the plasma membrane and to the nucleus with lesser amounts to the cytoplasm in leaf epidermal tissue (Figure 1 G-I). Expression of, in this case, the GFP-PDX2 protein fusion was verified by Western analysis, which demonstrated that the GFP-PDX2 protein was intact in
the leaves of the transformed plants. As PDX1 and PDX2 form a complex to function as PLP synthase, this group deliberately restricted their localization analysis to PDX2 only (Denslow et al., 2007). Some of these results contradict the studies reported within this thesis, which demonstrate that transient expression of the GFP fusion proteins of PDX1.1, PDX1.2, PDX1.3 and PDX2 in onion epidermal and Arabidopsis mesophyll cells give a signal exclusive to the cytoplasm (Chapter 2). Western blot analysis using a GFP antibody revealed that all the fusion proteins were intact. In addition, in experiments utilizing isolated pea chloroplasts the in vitro translated PDX1.3 protein was not taken up nor was the protein associated with the outer membrane of the chloroplasts (Titiz et al, unpublished observations) as suggested by Chen and Xiong (2005). As many forms of stress damage cellular membranes (Alexander-North et al., 1994; Havaux and Niyogi, 1999; Girotti and Kriska, 2004), localization of the PLP synthase in membranes might favour protection of the membrane by the vitamin produced in situ. The experimental approaches to localize the PDX proteins differ between the mentioned studies, however, common to all is the essential conclusion that vitamin B6 biosynthesis does not occur inside the plastids, but rather in the cytoplasm. PLP, in addition to its newly identified function as an antioxidant, is an important cofactor used in many cellular anabolic and catabolic functions within the cell many of which take place in the cytoplasm and thus, the cytoplasmic localization of the vitamin synthesizing machinery makes the vitamin freely available for at least these functional purposes within the cell. In summary, the cytoplasmic localization of the vitamin B6 synthesizing machinery can be safely concluded, but future studies involving the separation of cytoplasmic fractions will be instrumental to further define the site(s) of vitamin B6 biosynthesis.

Another recent discovery is the isolation and characterization of an Arabidopsis cDNA encoding a putative pyridoxine (pyridoxamine) 5-phosphate oxidase (AtPPOX, later named PDX3, Sang et al., (2007)). The putative PDX3 contains 3 distinct domains, an N-terminal putative chloroplast transit peptide followed by a so called ‘YjeN’ domain with unknown function and a C-terminal pyridoxine and/or pyridoxamine-oxidase domain (Sang et al. 2007). Heterologous expression of the C-terminal domain alone in E. coli gave a functional pyridoxine 5’ phosphate oxidase and this domain complemented a yeast strain deficient in PDX3. The actual localization of the protein has not yet been determined, but an in silico analysis predicts, with a high probability (80%) that PDX3 has an N-terminal chloroplast targeting sequence (Sang et al., 2007). If PDX3 is localized to the plastids, one must assume that pyridoxine-5’-phosphate and pyridoxamine-5’-phosphate are either produced inside the chloroplast or are transported into this organelle. If the salvage pathway is indeed associated with the chloroplast, the oxidation reaction would make the active form of the vitamer available in an organelle which is known as a site of ROS production.
Expression and regulation of the *PDX* homologs in *Arabidopsis*

5.0.1 Expression of the PDX proteins

*PDX1.1, PDX1.2* and *PDX1.3* are expressed in various organs and vary depending on the developmental stage of *A. thaliana* (Chapter 3). At the transcript level, the expression appears to be strongest at younger stages, followed by a gradual decline until senescence. This pattern is most pronounced in the case of *PDX1.3*. In contrast, the *PDX1.2* transcript accumulates during senescence. Generally, there is a good correlation between the expression pattern of *PDX1.1 and PDX1.3*, while *PDX1.2* shows a pattern quite different from that of either of the other genes. However, there are also differences in the expression between *PDX1.1 and PDX1.3* (e.g. specific expression of *PDX1.3* in pollen). The relative transcript abundance of *PDX1* and *PDX2* genes in *A. thaliana* rosettes was also examined by Denslow et al. (2007). Similar to our findings, *PDX1.1 and PDX1.3* transcripts were found to be present in high amounts, while the *PDX1.2* transcript was present only at a low level, less than 0.5% of either *PDX1.1 or PDX1.3*. The *PDX2* transcript was present at a low level as well, but was still more prevalent than that of *PDX1.2* (Denslow et al., 2007). A survey of the expression of the *PDX1* and *PDX2* genes using the *Arabidopsis* microarray database, Genevestigator (Zimmermann et al., 2004) supported these two analyses. Wagner et al. (2006) used RT-PCR, as well as analysis of the expression of promoter –GUS fusions of the *PDX* genes (pPDX1.1::GUS, pPDX1.3::GUS), to determine their specific expression. *PDX1.3* was found to be expressed in flowers, shoots, and leaves, but only very weakly in root tissue. A similar expression pattern was observed for *PDX1.1*, whereas *PDX1.2* was expressed at low levels in leaves, with clearly stronger expression in flowers and roots. In comparison, *PDX2* was strongly expressed in all tissues examined. pPDX1.1:GUS and pPDX1.3:GUS constructs gave overlapping expression patterns, with high activity in leaves and weak activity detected in roots. In flowers, pPDX1.1:GUS lines exhibited a strong dot-like staining in pistils. In comparison, promoter pPDX1.3:GUS lines showed strong staining in anthers and sepals. Furthermore, pPDX1.3: GUS lines expressed GUS only at the top and base of differentiated siliques, with no staining detectable in the seeds at all stages examined. In pPDX1.2:GUS seedlings, there was weak expression in the leaf vascular tissue, but generally in the pPDX1.2:GUS lines there was a root tip–specific expression pattern (Wagner et al., 2006). Chen and Xiong (2005) found by Northern blot analysis that *PDX1.3* was expressed in all plant organs including stem, leaf, flower and siliques, but to a lesser extent in the root. Interestingly, when they analysed pPDX1.3:GUS, the promoter directed GUS expression in roots, and the highest expression was observed near the
root tips and in the stele of rapidly growing roots. However, the pPDX1.3-GUS signal was very faint or not detectable in the root tips. GUS expression was also observed in root hairs. In cotyledons, pPDX1.3-GUS activity was also observed, with especially strong signals in guard cells of cotyledons and vascular tissues of stems. In floral organs, pPDX1.3-GUS expression was observed in mature pollen grains and filaments. The most pronounced pPDX1.3-GUS staining was detected around cutting sites, suggesting that wounding may induce PDX1.3 expression. Common to all the recent discoveries, and in contradiction to Chen and Xiong’s findings (root tissue expression of PDX1.3) PDX2, PDX1.1 and PDX1.3 are expressed mainly in the above ground organs, and PDX1.2 gene expression is generally very low compared to that of all the other three and the transcript is present mostly in the root tissue.

5.0.2 The physiological role of de novo and salvage pathway proteins in vitamin B6 homeostasis

The spatial and temporal expression patterns of PDX1.1 and PDX1.3 at the transcription and protein level largely overlap, but PDX1.3 is more abundant than PDX1.1 (Chapter 3). In order to characterize vitamin B6 biosynthesis mutants, we obtained A. thaliana mutant strains with DNA insertions in the coding regions of the functional PDX1 homologues, which abolished the respective PDX transcript and protein. When the phenotypes of these plants were analysed, the above ground organs of the soil grown pdx1.1 plants appeared to develop similar to wild type plants (Titiz et al., 2006), but the rosette leaves of pdx1.3 were slightly chlorotic (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006). When grown in sterile culture in the absence of pyridoxine, pdx1.3 seedlings showed reduced shoot growth and a distinct pale green phenotype, most apparent at the newly emerging part of the leaf, while pdx1.1 seedlings looked normal. Under the same conditions, the length of the primary root of pdx1.3 was severely reduced (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006), while the length of pdx1.1 roots was between that of the wild type and pdx1.3. Over an extended period, root growth of pdx1.3 was more retarded than shoot growth. Phenotypes of the pdx1 mutants correlated with their vitamin B6 content, such that pdx1.3, which has a more pronounced root and shoot phenotype, also had less vitamin B6 in its root and shoot. Supplementation with pyridoxine abolished all the growth defects of the pdx1 plants, confirming that the observed phenotype was due to vitamin B6 deficiency. The fact that the phenotype of the mutant plants is much less pronounced in soil as compared to sterile medium in the absence of vitamin B6, and
especially the restoration of the growth of the roots of *pdx1.3*, implies that in *Arabidopsis* there is an uptake system in place for the vitamin and an unidentified source of the vitamin in soil.

The dark grown etiolated *pdx1* seedlings in sterile culture without vitamin B6 supply had a reduced apical hook curvature which was more exaggerated in the *pdx1.3* as compared to the *pdx1.1* seedlings. A phenotypical characteristic of mutants defective in ethylene perception and/or biosynthesis is the lack of the apical hook curvature. As the ethylene precursor, ACC, requires a PLP-dependent enzyme for its biosynthesis, i.e. ACC synthase, the restoration of the etiolated phenotypes of *pdx1* in the presence of either ACC or of vitamin B6, confirmed that the observed etiolated phenotype was directly due to vitamin B6 deficiency.

The salvage pathway of vitamin B6 biosynthesis, which has been described in bacteria, fungi and plants, requires the activity of PdxH (PDX3) and PdxK, a vitamin B6 oxidase, and a vitamin B6 kinase, respectively (Yang et al., 1996; Mittenhuber, 2001). Until recently, very little was known about the salvage pathway in plants. In 2002, the *Arabidopsis thaliana* salt-sensitive mutant *salt overly sensitive4* (*sos4*) was described (Shi et al., 2002; Shi and Zhu, 2002), which carries a mutation in one of the salvage pathway genes, i.e. pyridoxal kinase (PdxK). In the same study, it was shown that loss of *SOS4* caused an altered root hair development along with increased sensitivities towards Na⁺, K⁺ and Li⁺ ions, which correlated with an altered cellular ion content. Very recently, Daub and co-workers characterized two independent *sos4* lines and concluded that the *sos4* phenotype is very similar to that of the *pdx1.3* mutant. Not only did *sos4* have short roots in the absence of pyridoxine, but in addition to NaCl sensitivity, the roots of *sos4* plants were hypersensitive to sucrose, the plants were chlorotic and had a visibly smaller shoot size when grown under normal conditions (Gonzalez et al., 2007). In the same study, the authors characterized a *pdx3* mutant. Although shoot and root growth were decreased in the *pdx3* mutant and the total vitamin B6 content was reduced compared to that of wild type plants, these mutant lines were not as strongly affected as *sos4* mutant plants. The growth of *pdx3* mutant plants was not affected by sucrose and NaCl, but they were incapable of increased growth under high light conditions, and were decreased in *PDX1* activity, as compared to both wild type and *sos4* mutant plants. The activity of PDX1 was measured in crude extracts of the mutant lines and wild type based on an enzyme assay previously described (Herrero et al., 2007). The less dramatic phenotype of *pdx3* may be due to the fact that these mutants have only a reduced PDX3 activity, but are not completely deficient in this enzyme. The *sos4* plants have an 8 times higher PLP level, when compared to the wild type. The similarities between *sos4* and *pdx1.3* phenotypes indicate that, in the absence of SOS4, the salvage pathway PDX3 protein and *de novo* PDX1.3 and PDX2 biosynthesis machinery can generate excessive amounts of vitamin B6, but this level still cannot rescue the short root and chlorotic phenotype of *sos4*. One possible way to explain this observation is an increased activity of the *de novo* pathway. In the
same publication, the gene regulation experiments did not support this hypothesis, but the PDX1 enzyme activity did correlate with the 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 wild type, indicating greater activity of both salvage and *de novo* pathways in this mutant. It has not yet been determined which type of vitamer is available in soil, but it is more likely to be the un-phosphorylated form. The increased levels in PN and PM in the *sos4* mutant plants by HPLC analysis support the hypothesis that SOS4 can also phosphorylate PN and PM, consistent with SOS4 encoding a PL/PN/PM kinase. If PN and PM are taken up and phosphorylated by the SOS4, this would imply that when the vitamin is available in the soil it is taken up and converted to its active form by the SOS4 protein which is localized in the root. SOS4 is highly expressed in root tissues i.e. root hairs (Shi and Zhu, 2002). However, when the *de novo* vitamin supply is scarce, the vitamin is supplied by the SOS4 salvage pathway. When the *de novo* pathway of vitamin B6 synthesis is compromised, as in *pdx1.3*, and when there is no exogenous vitamin B6 supply, the *sos4* or *pdx1.3* phenotype develops. However, when SOS4 is mutated, the *de novo* pathway machinery can synthesize the vitamin in the above ground organs. The increase in PLP in *sos4* mutant plants suggests in addition to the increased activity of the *de novo* bioynthesis, the presence of an additional kinase in *Arabidopsis* that would preferentially phosphorylate PL, hence the increase in PN and PM and the lack of over accumulation of PL.

A physiological implication of the lack of vitamin B6 homeostasis can be observed in the *pdx3* mutant, which is incapable of increased growth under high light conditions (Gonzalez et al., 2007). PDX3 has a chloroplast targeting N terminal transit peptide. A possible explanation is that during high light conditions, excess ROS are produced and the vitamin B6 supply generated the pyridoxal kinase salvage synthesis in the chloroplast is used up by the excessive ROS. That is to say that under ROS stress, the available vitamin B6 in the chloroplast may preferentially be required for antioxidant supply, and the rate of consumption of vitamin B6 may exceed that of its supply and hence in the *pdx1.3* mutant there will not be enough vitamin B6 left for other cellular functions.

Surprisingly, Gonzalez et al. (2007) do not mention the previously reported root hair phenotype of the *sos4* mutant (Shi and Zhu, 2002). It is worth noting that during the course of this thesis we observed in the T4 generation that the *pdx1.3* and *pdx1.1* plants lacked root hairs in the maturation zone of the primary root. This phenotype was more evident in the *pdx1.1* plants as they had a longer primary root, but it was not observed again in the progeny. The reason for this is not known at present.

In the case of the double *pdx1.1* and *pdx1.3* mutant lines, failure to identify seedlings homozygous for T-DNA insertions in the progeny of the crosses, and the arrest of embryo
development observed in 1/16 of \( \text{pdx1} \) heterozygous plants indicated that \( \text{pdx1.1} \) and \( \text{pdx1.3} \) double homozygous plants are not viable. The arrest of embryo growth at the globular stage of development was remarkably similar to that of the \( \text{pdx2} \) mutant (Chapter 2). This might indicate that at the globular stage of embryo development an enzymatic activity which requires PLP as a cofactor is essential. In flowering plants, embryogenesis is a highly orchestrated process of cell division, differentiation, growth, and pattern formation (Mansfield and Briarty, 1991; Mayer and Jürgens, 1998). A minimal set of ~750 non-redundant genes is thought to be required in \textit{Arabidopsis thaliana} to coordinate these embryo developmental events (McElver et al., 2001; Tzafrir et al., 2003). In the process of identifying all \textit{Arabidopsis} genes essential for seed development, an initial set of ~250 genes essential for normal embryo development has been reported (McElver et al., 2001; Tzafrir et al., 2003). These genes function in various processes, including cell growth, metabolism, transport, transcription, and translation and are especially required at the switch from heterotrophy to autotrophy, which is thought to occur at the globular stage of embryo development. As PLP is a widely used cofactor in some of these processes, it is plausible that its shortage leads to the observed arrest at the globular stage. Table 1 summarizes some of the globular stage arrest mutants. Even though B6 biosynthesis of these mutants is not different from that in the WT, the normal, wild type functioning of these pathways would also require PLP synthesis in the first place. Hence, some analogy can be built such that PLP is required in the upstream biosynthesis pathways of the mentioned proteins and any downstream event mutations would also lead to a similar phenotype. During embryo growth, cell polarity (apical and basal) is established by an auxin gradient during embryogenesis (Friml et al., 2006). \textit{Arabidopsis thaliana} hypocotyl segments produce adventitious roots in response to exogenously supplied auxin; \textit{root primordium defective 1} (\textit{rpd1}) is a temperature-sensitive mutant isolated on the basis of impairment in this phenomenon (Konishi and Sugiyama, 2006). Disruption of the \textit{RPD1} gene by a T-DNA insertion caused embryogenesis arrest at the globular stages (Konishi and Sugiyama, 2006). As auxin is thought to require PLP as a cofactor for its biosynthesis, it is not surprising that the presumed lack of auxin (due to a shortage of its precursor tryptophan which requires PLP for its synthesis) would affect the growth during embryogenesis and lead to an arrest at the globular stage i.e. before the establishment of the longitudinal axis. The lethal phenotype also indicates that the transport of nutrients from surrounding maternal tissues is not adequate to rescue this phenotype or does not take place at all. Especially the early stages of embryo development require high amounts of energy for rapid growth of the embryo. Embryo development depends on the import of carbohydrates and amino acids supplied by the maternal tissue via the phloem. Several amino acid transporters have been reported to be expressed during seed and silique development in \textit{Arabidopsis thaliana}. The
embryo lethal phenotypes of the amino acid transporters *app8* and *ptr2* indicate that embryo development at early stages requires nutrients from the surrounding maternal tissues (Table 1). In fact the embryo lethal phenotype of mutants with defects in vitamin B6, or amino acid transporters are in line with the assumption that the surrounding maternal tissues can supply the

<table>
<thead>
<tr>
<th>Mutants with arrest in globular stage</th>
<th>Protein function / characteristics</th>
<th>Their PLP requirement for</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>root primordium defective 1 (<em>rpd1</em>)</td>
<td>impairment in cell polarity by an auxin gradient during embryogenesis</td>
<td>Auxin biosynthesis requires PLP as a cofactor</td>
<td>(Konishi and Sugiyama, 2006)</td>
</tr>
<tr>
<td>amino acid permease 8 (<em>app8</em>)</td>
<td>function in uptake of amino acids into the endosperm, mutant sensitive to lack of nitrogen supply</td>
<td>Nitrogen is first assimilated into the amino acids Glu and Gln by glutamine synthetase / glutamate synthase, and later into Asp and Asn by aspartate aminotransferase (AAT) and asparagine synthetase (AS). AAT as well as glutamate synthase require pyridoxal phosphate as a cofactor.</td>
<td>(Schmidt et al., 2007)</td>
</tr>
<tr>
<td>transporter for dipeptides (<em>ptr2</em>)</td>
<td>Transporter of dipeptides expressed in young siliques, mutant sensitive to lack of nitrogen</td>
<td>same as above</td>
<td>(Song et al., 1996)</td>
</tr>
</tbody>
</table>

*Table 1.* Mutants identified by arrest at the globular stage of embryogenesis. The corresponding proteins may indirectly require PLP for proper functioning.

developing embryo with required nutrients only at early stages of development before the switch from heterotrophy to autotrophy. The lethal phenotype of the double mutant (*pdx1.1/pdx1.3*) is consistent with the conclusion that PDX1.2 is not functional in vitamin B6 biosynthesis and hence cannot compensate for the absence of PDX1.1 and PDX1.3. The arrested *pdx2* embryos have been rescued by addition of vitamin B6 to the growth medium (Tambasco-Studart et al., 2007), further supporting the notion that the arrested phenotype is due to a shortage in vitamin B6 supply.

Of the plants carrying only a single copy of either functional *PDX1* gene, the phenotype of a single copy *PDX1.1* plant is more severe than that of a plant having a single *PDX1.3*. The single copy *PDX1.1* plants, in comparison to single copy *PDX1.3*, were also characterized by a
lack of elongation at the tip and the base of the differentiated siliques. The PDX1.3 promoter directed expression of GUS (Wagner et al., 2006) was observed at the tip and the base of the siliques, hence the lack of development in those parts could be the result of the absence of the gene product. Generally, the transcript of PDX1.3 was observed to be more abundant than that of PDX1.1. In corroboration of this, a plant with a complete PDX1.3 knockout and only a single copy PDX1.1 is more handicapped than a plant with a complete PDX1.1 knockout and a single copy PDX1.3. In addition, the timing of the switch from vegetative to reproductive growth was dependent on the presence of the single copy PDX1.3. Thus, PDX1.3 is more of a prerequisite than PDX1.1. These observations suggested a dosage effect hypothesis, where the extent of the phenotype correlated with the vitamin B6 content. Moreover, the greater lack of vitamin B6 and hence, the ethylene precursor, ACC, in single copy PDX1.1 plants, may make these plants more sensitive to sugars than the single copy PDX1.3 plants. In conclusion, the detailed examination of the single mutants as well as the double mutants which carry only a single functional copy of either gene indicates that although these genes are partially redundant in vitamin B6 synthesis, PDX1.3 is more a prerequisite.

Possible explanations for the pdx1.3 mutant phenotype

A possible explanation for the chlorotic leaf phenotype of the pdx1.3 mutant could be that it harbours defects in chlorophyll biosynthesis, which requires the activity of the PLP-dependent δ-aminolevulinate synthase (Papenbrock and Grimm, 2001). On the other hand and interestingly, when the root elongation rate of the pdx1.3 mutant was analyzed in the absence of exogenous vitamin B6, in greater detail (Figure 2), it was observed that, for the first 4 to 7 days after germination, the roots of the pdx1.3 mutant were severely retarded in growth, whereas the roots of pdx1.1 elongated at a constant yet slower rate than those of the wild type. Around the 7th day after germination, the pdx1.3 roots start to elongate, at a constant, but slightly slower rate than those of pdx1.1 and the WT (Figure 2). This developmental switch which initiates the pdx1.3 root to elongate at a faster rate coincides with the developmentally regulated switch of auxin supply to roots within the first ten days of growth (Bhalerao et al., 2002). It has been demonstrated that in the first four to seven days after germination (DAG), roots are highly dependent on shoot-derived auxin, whereas this is not the case with older seedlings (seven to ten DAG) (Bhalerao et al., 2002). Based on these observations, the following scenario may be visualised. When PDX1.3 is knocked out, the level of vitamin B6 generated by PDX1.1 in the shoot tissue may not be adequate to meet the vitamin B6 demand for growth functions, for antioxidant generation under photo-oxidative stress under normal light growth conditions, and in addition, to support the biosynthesis of auxin in shoot and root tissue. Therefore, there is no or minimal growth of the root during the first 3-6 days after germination. But at 7 DAG, the root
tissue starts generating its own source of auxin, and hence is no longer constrained by the shoot supply of auxin, and thus the \textit{pdx1.3} root starts to elongate. In \textit{pdx1.3}, the supply of vitamin B6 for the biosynthesis of auxin, also in the root tissue, is quite scarce, which is demonstrated by the slope of the root elongation rate after the 7\textsuperscript{th} day, but nevertheless, it stays constant for the remaining period of its growth. The PDX1.1 knock outs on the other hand have a better supply of vitamin B6, hence the absence of chlorotic leaves and the normal growth of shoot tissue, and root elongation proceeds similar to wild type albeit at a slower rate.

The rate of auxin synthesis differs between the different organs of a plant. It was documented that IAA levels differ by more than two orders of magnitude between generative organs and non-generative organs, especially growing siliques. Within leaves of a rosette, there is a pronounced gradient in IAA concentrations from the youngest, expanding leaves (highest) to the fully grown source leaves (lowest) (Müller et al., 2002). Within a leaf, growth zones and central veins are the areas most rich in IAA (Müller et al., 2002). There is a striking correlation between the areas with the highest auxin levels and the highly chlorotic regions observed in the \textit{pdx1} plants, such that in the single copy \textit{PDX1.1} plants newly emerging tissue within the young rosette, and in particular the central vein of the leaf, is much more chlorotic than the rest of the plant. On the other hand, while the single copy \textit{PDX1.3} plants are generally somewhat pale, there is no distinct gradient pattern. A correlation between the sites of highest auxin biosynthesis and lack of chlorophyll in the single copy \textit{PDX1.1} plants is not likely to be coincidental. A

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Root growth of the \textit{pdx1} mutants and the WT. The roots were periodically measured the same hour of each day for two weeks. The seeds had been plated on medium lacking vitamin B6.}
\end{figure}
A possible explanation is that, when the vitamin B6 content is low, there are not sufficient amounts to support auxin biosynthesis as well as heme (chlorophyll) biosynthesis. With regard to growth in the single copy PDX1.3 plants, growth retardation is not as severe as in complete pdx1.3 knockout plants, possibly because in single copy PDX1.3 plants the rate of vitamin B6 biosynthesis is sufficient to supply auxin for the first 7 DAG (data not shown). In addition, the level of vitamin B6 is sufficient to support both auxin and heme biosynthesis; thus no distinct chlorotic pattern is observed.

**Regulation of the PDX1 genes**

The regulation of the PDX1 genes was briefly addressed in chapter 4. Recent results on the regulation of PDX1 and PDX2 will be discussed here. Vitamin B6 has been shown to quench ROS and protect other molecules from oxidative damage (Bilski et al., 2000; Jain and Lim, 2001; Stocker et al., 2003; Kannan and Jain, 2004; Chen and Xiong, 2005; Danon et al., 2005; Denslow et al., 2005). Chen and Xiong (Chen and Xiong, 2005) observed that the pdx1.3 mutant was sensitive to salt, UV and singlet oxygen. In tobacco, the vitamin B6 genes are regulated in such a way that during bacterially induced HR (Hypersensitive Response) the PDX1.1 and PDX1.3 genes are strongly downregulated (Denslow et al., 2005). Externally applied vitamin B6 delayed HR. From this finding, the authors concluded that vitamin B6 counteracts plant defense responses against biotic pathogens. However, as the observed downregulation of the PDX1 genes was only analyzed at the RNA level and as the delay of the HR by exogenous vitamin B6, was only done at a single concentration the data are not sufficient to support such a conclusion. On the other hand, Chen and Xiong (2005) cited microarray data indicating that the PDX1 genes are not regulated by stress conditions. However, Denslow et al. (2007) disputed these results and concluded that the PDX1 genes are regulated by diverse abiotic conditions. Both groups concurred that the pdx1.3 mutant is sensitive to salt, and confirmed the earlier results of Brosche et al. (2002) that the PDX1 transcript is upregulated by UV light. Chen and Xiong (2005) and Denslow et al. (2007) also documented that the pdx1.3 mutant is sensitive to paraquat, which generates the superoxide anion. These recent findings support our initial conclusions and highlight the importance of vitamin B6 in stress responses.

Contrary to the coding regions of the PDX1 genes, the respective 1.4 kp upstream regions are highly dissimilar. Especially with respect to the putative cis regulatory elements, the three promoters differ considerably (Table 2).
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>PDX1.1</th>
<th>PDX1.2</th>
<th>PDX1.3</th>
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<tr>
<td>Auxin</td>
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</tr>
</tbody>
</table>

**Table 2.** Potential binding sites for stress-responsive regulatory elements. Motifs were identified in the 1.4Kb regions upstream of the predicted start codon ATG. Number identified for each motif is shown in parenthesis. Adapted from Denslow (2005)

The *PDX1* promoters have been reported to direct expression at distinct locations within the plant (Chen and Xiong, 2005; Wagner et al., 2006). The **PDX1.1** and the **PDX1.3** promoters are mainly active in above ground organs of *Arabidopsis*, whereas the **PDX1.2** promoter is active in unique parts of the plant where neither of the other two **PDX1** promoters is active. To assess the
regulation of PDX1 genes in stress conditions, we conducted a survey of the response viewer programme of the Arabidopsis microarray database, Genevestigator (Zimmermann et al., 2004). The data (Table 3) indicate that of the three PDX1's, PDX1.2 is by far the most sensitive to the majority of the given stress conditions. Norflurazon, a herbicide inhibiting carotene and linolenic acid biosynthesis in plants, thus producing oxidative stress, caused an almost 19 fold increase in PDX1.2 transcript abundance; furyl acrylate ester, which inhibits auxin signalling in Arabidopsis (Armstrong et al., 2004) caused an almost 7 fold increase in PDX1.2 transcription; Boron accumulation which in turn causes oxidative stress (Hayes and Reid, 2004), caused a 5 fold increase in the PDX1.2 transcript; H₂O₂ application resulted in a 4.5 fold increase, while CO₂ stress, known to induce ethylene synthesis (Mathooko et al., 1999) resulted in a 4.5 fold increase. The dramatic response of the PDX1.2 transcript level to various external stressors is indicative of a role in the response to stress. On the other hand, the PDX1.1 transcript was induced during senescence, whereas the expression of PDX1.2 and PDX1.3 was remarkably reduced. This, with a somewhat lower degree of correlation, is corroborated by our findings (Chapter 3). Nematodes caused a significant increase in the PDX1.2 and PDX1.3 transcripts. Application of the synthetic auxin, 2,4-D, and of ethylene led to a significant increase in the PDX1.2 transcript in addition to the PDX1.3 transcript whereas the PDX1.1 transcript remained unchanged Genevestigator (Zimmermann et al., 2004). Independently the response of PDX1.3 to ethylene has been documented in chapter 4. These responses may be explained by the presence of the putative AuxRE as well as ERE elements in both the PDX1.2 and the PDX1.3 promoters. PDX1.2 and PDX1.3 transcripts also increase under under heat stress (18 fold in the case of PDX1.2, 5 fold in the case of PDX1.3, whereas there was no such increase observed for PDX1.1) Thus, there may be a hint that PDX1.2 and PDX1.3 are co-regulated under certain conditions. One way to test if there is a possible co-regulation of PDX1.2 and PDX1.3 under these conditions would be to fuse the promoters of the respective genes to genes encoding fluorescing proteins with different excitation wavelengths and thereafter monitor the fluorescence during and after stress.
Table 3 The response of $PDX1.1-3$ transcripts to given stressors, analyzed by Response Viewer (Genevestigator: www.genevestigator.eth.ch). The numbers indicate fold change values relative to Col-0 wild type. The color coding reflects the up (green) or down (red) regulation of the respective transcripts.
General conclusion and outlook

It has been shown in this thesis that, contrary to what has been assumed, vitamin B6 biosynthesis in plants occurs independently of the compound DXP. Instead, vitamin B6 is synthesized from intermediates of the pentose phosphate pathway (i.e. ribose 5′-phosphate or ribulose 5′-phosphate) and glycolysis (i.e. glyceraldehyde 3′-phosphate or dihydroxyacetone phosphate). In addition, the pathway was found to be cytosolic rather than plastidial. Of the three PDX1 proteins identified in *Arabidopsis thaliana*, PDX1.1 and PDX1.3 are active in PLP biosynthesis, whereas PDX1.2 which shows less homology than PDX1.1 to PDX1.3, is not catalytically active. An *in silico* analysis suggests that temporal and spatial expression patterns of *PDX1.2* transcripts may be quite different from those of *PDX1.1* and *PDX1.3* and that *PDX1.2* transcript levels are lower in abundance in comparison with the other *PDX1* transcripts. The exact function of PDX1.2 still remains to be elucidated but it may play a regulatory role, which could be the basis of a future investigation. PDX1.2 knock-outs or knock downs generated with RNA interference technology may provide some information on the role of this protein. As a final endeavour to understand the distinct short root phenotype of the *pdx1.3*, the auxin levels in the root and shoot tissue of seedlings grown with and without vitamin B6 can be studied using the unique multiplex GC-MS/MS technique of Müller et al. (2002). The IAA measurement at specific time points, i.e. 4 and 10 DAG, can provide information, whether the delayed start of root growth in *pdx1.3* is due to the shortage of the shoot derived IAA.

As an indication of the distribution of free auxin in the *pdx1.3* plants, the synthetic DR5 promoter which responds to auxin can be analyzed. The DR5 auxin responsive promoter is already available fused to either GFP or the GUS reporter gene (obtained from J. Friml; Zentrum Molekularbiologie der Pflanzen, Universität Tübingen).

In an effort to understand the distinction between the *pdx1.1* and *pdx1.3* mutant phenotypes, we have discovered that there are certain unique cis acting elements in the promoters of the two genes. In particular, the promoter region of *PDX1.3* has a putative auxin (AuxRE) and ethylene response element (ERE) not present in the promoter of *PDX1.1*. The relevance of these elements and their regulatory role can be investigated by promoter deletion constructs.
List of Abbreviations

ACC: 1-Aminocyclopropane-1-Carboxylic Acid
AuxRE: Auxin Response Element
DAG: Days After Germination
DXP: 1- deoxy- D-xylulose 5-phosphate
DXS: DXP synthase
ERE: Ethylene Response Element
IPP: isopentenyl diphosphate
PLP: pyridoxal-5’-phosphate
PMP: pyridoxamine-5’-phosphate
PNP: pyridoxine-5’-phosphate
PPOX: pyridoxine (pyridoxamine) 5’-phosphate (PNP/PMP) oxidase
ROS: reactive oxygen species
SOS4: Salt Overly Sensitive 4
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Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF (2000) Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem. Photobiol. 71: 129-134


Chen H, Xiong L (2005) Pyridoxine is required for post-embryonic root development and tolerance to osmotic and oxidative stresses. Plant J. 44: 396-408


Gonzalez E, Danehower D, Daub ME (2007) 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 B6 salvage pathway. Plant Physiol. 145: 985-996


### Appendix

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Publications:


Meeting abstract:


(*) Equal contribution by these authors