Running head: Pyridoxal 5'-phosphate synthase from *Arabidopsis thaliana*

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Journal research area: Biochemical processes and macromolecular structures
Functional analysis of PDX2 from *Arabidopsis thaliana*, a glutaminase involved in vitamin B6 biosynthesis.

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FOOTNOTES

1This work was supported by the ETH Zurich (grant number 0094/41-2703.5).

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‡ The abbreviations used are: DHAP, dihydroxyacetone phosphate; DXP, deoxyxylulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; PLP, pyridoxal 5′-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate.
ABSTRACT
Vitamin B6 is an essential metabolite in all organisms being required as a cofactor for a wide variety of biochemical reactions. De novo biosynthesis of the vitamin occurs in microorganisms and plants, but animals must obtain it from their diet. Two distinct and mutually exclusive de novo pathways have been identified to date, namely deoxyxylulose 5-phosphate (DXP)-dependent, which is restricted to a subset of eubacteria, and DXP-independent, present in archaea, fungi, plants, protista and most eubacteria. In these organisms, pyridoxal 5'-phosphate (PLP) formation is catalyzed by a single glutamine amidotransferase (PLP synthase) composed of a glutaminase domain, PDX2, and a synthase domain, PDX1. Despite of plants being an important source of vitamin B6, very little is known about its biosynthesis. Here, we provide information for Arabidopsis thaliana. The functionality of PDX2 is demonstrated, using both in vitro and in vivo analyses. The expression pattern of PDX2 is assessed at both the RNA and protein level providing insight into the spatial and temporal pattern of vitamin B6 biosynthesis. We then provide a detailed biochemical analysis of the plant PLP synthase complex. While the active sites of PDX1 and PDX2 are remote from each other, co-ordination of catalysis is much more pronounced with the plant proteins than its bacterial counterpart, Bacillus subtilis. Based on a model of the PDX1/PDX2 complex, mutation of a single residue uncouples enzyme coordination and in turn provides tangible evidence for the existence of the recently proposed ammonia tunnel through the core of PDX1.
INTRODUCTION

Vitamin B6 is the generic name for pyridoxal, pyridoxol, pyridoxamine and their respective 5’-phosphorylated forms. This vitamin is essential for all organisms and its functional forms, pyridoxal 5’-phosphate (PLP) and pyridoxamine 5’-phosphate, play a crucial role in a broad range of biochemical reactions. Furthermore, vitamin B6 has recently been identified as a potent antioxidant (Ehrenshaft et al., 1999; Osmani et al., 1999; Bilski et al., 2000). Prokaryotes, fungi, protista and plants are able to synthesize the vitamin de novo, but animals must obtain it from dietary sources. The biosynthetic pathway of vitamin B6 might therefore be an important target for drug design (Wrenger et al., 2005; Gengenbacher et al., 2006).

Two distinctive pathways for its de novo synthesis have been identified to date. In the DXP-dependent pathway (Tambasco-Studart et al., 2005), present in only a small subset of eubacteria, pyridoxal 5’-phosphate biosynthesis is mediated by the proteins PdxA and PdxJ from the precursors deoxyxylulose 5-phosphate (DXP) and 4-phosphohydroxy-L-threonine (Spenser and Hill, 1995; Canet al., 1999; Laber et al., 1999; Drewke and Leistner, 2001). The second pathway, referred to as DXP-independent (Tambasco-Studart et al., 2005), was only recently discovered and appears to be present in all archaea, fungi, plants and most eubacteria (Ehrenshaft et al., 1999; Mittenhuber, 2001; Tanaka et al., 2005). The cofactor form of the vitamin, pyridoxal 5’-phosphate (PLP), is synthesized directly from either ribose 5-phosphate (R5P) or ribulose 5-phosphate (Ru5P), in combination with either glyceraldehyde 3-phosphate (G3P) or dihydroxyacetone phosphate (DHAP), and glutamine as the N-donor (Burns et al., 2005; Raschle et al., 2005) (Scheme 1). Remarkably, the reaction is catalyzed by a single glutamine amidotransferase (PLP synthase), composed of the glutaminase subunit, Pdx2, and the synthase or acceptor subunit, Pdx1.

Glutamine amidotransferases in general provide an impetus for research not only because they play a central role in metabolism, through the incorporation of nitrogen into amino acids, amino sugars, nucleotides, co-enzymes and antibiotics, but also because they coordinate catalysis at distinctive, and in all cases examined thus far, remote active sites (Myers et al., 2005; Strohmeier et al., 2006; Zein et al., 2006). Substrate (ammonia) channeling between the two sites is achieved via a protein tunnel between the glutaminase and synthase active site, respectively (Zalkin and Smith, 1998; Raushel et al., 1999). Furthermore, in many cases the glutaminase is activated only upon binding of the substrates in the associated synthase domain, thus preventing futile hydrolysis of glutamine (Zalkin and Smith, 1998; Bera et al., 2000). Thus far, neither of these phenomena has been observed for PLP synthase making it somewhat of an enigma in the glutamine amidotransferase family.
However, the structure of bacterial PLP synthase has recently been solved (Strohmeier et al., 2006; Zein et al., 2006) and demonstrates activation of Pdx2 when in complex with Pdx1. Furthermore, a methionine rich hydrophobic tunnel has been proposed for the transfer of ammonia between the two active sites of bacterial PLP synthase (Strohmeier et al., 2006).

The polymorphic synthetic ability of PLP synthase has attracted considerable interest. PLP synthases from various organisms are being studied in the expectation that questions unanswered for one of the synthases can be revealed in another (Belitsky, 2004; Dong et al., 2004; Burns et al., 2005; Chen and Xiong, 2005; Raschle et al., 2005; Tambasco-Studart et al., 2005; Wrenger et al., 2005; Gengenbacher et al., 2006; Wagner et al., 2006). Currently, the functionality of both the Pdx1 and Pdx2 subunits as a complex in vitro and reconstitution of PLP biosynthesis have been demonstrated for the bacterium Bacillus subtilis and the apicomplexan Plasmodium falciparum (Burns et al., 2005; Raschle et al., 2005; Gengenbacher et al., 2006). In the model plant Arabidopsis thaliana, we have recently identified three homologs of PDX1, named PDX1.1, PDX1.2 and PDX1.3, and one homolog of PDX2 (Tambasco-Studart et al., 2005). Even though the disruption of PDX2 was shown to be lethal for the plant (Tambasco-Studart et al., 2005), the glutaminase activity of this protein and its direct correlation with vitamin B6 biosynthesis could not be demonstrated in that study. Moreover, the enzymatic activities of the PDX1 proteins could be verified in vitro only by using ammonium sulfate as the nitrogen source and in the absence of PDX2, which does not reflect the presumed in vivo scenario.

In this study, we provide a detailed description of the role of PDX2 in the biosynthesis of PLP in the model plant Arabidopsis thaliana. A comprehensive expression analysis at both the RNA and protein level and its comparison with that of PDX1 has allowed us to describe the spatial and temporal formation of vitamin B6 in plants. The functionality of PDX2 in vitamin B6 biosynthesis has been established in vivo by the ability of the vitamin to rescue pdx2 seeds, and in vitro by the discovery of glutaminase activity of PDX2 in the presence of PDX1. A unique feature of plant PDX2 proteins is a C-terminal extension of approximately thirty amino acids that we show here to be important for catalysis. We also report a detailed biochemical analysis of the PLP synthase protein complex, and compare it mechanistically to its prokaryotic counterparts. Lastly, we provide evidence that the co-ordination, or coupling, between the glutaminase and synthase domains is more pronounced with the plant as compared to the bacterial proteins, and that this may be due to an alteration at the base of the proposed ammonia tunnel in Pdx1.
RESULTS

In vivo evidence for the involvement of PDX2 in vitamin B6 biosynthesis in planta

We recently reported the isolation and characterization of the mutants \textit{pdx2.1} (SALK\_072168) and \textit{pdx2.2} (53-2381-1) from \textit{Arabidopsis thaliana} (Tambasco-Studart et al., 2005). These mutants are arrested at the globular stage of embryo development, a notoriously difficult stage from which to rescue the seed (Patton et al., 1998), thus complicating analyses of gene functionality. After many previous unsuccessful attempts, we can now provide direct evidence for the involvement of PDX2 in vitamin B6 metabolism in planta. Culturing of albino seeds produced by \textit{PDX2/pdx2} plants (Fig. 1A) in the presence of vitamin B6, as described under methods, led to growth after 14 days (Fig. 1B right side), albeit in a heterogeneous fashion (Fig. 1C). \textit{pdx2} seeds did not respond on medium lacking the vitamin, whereas WT seeds developed and germinated (Fig. 1B left side). Successful rescue of \textit{pdx2} was only achieved if seeds were cultured at the time they had reached the globular stage of development, and under low light conditions. Merely watering, spraying or even vacuum infiltration of the vitamin did not result in an advancement of embryo development in the albino seeds beyond the globular stage. After 50 days, the teratomata that had resulted from the described successful rescue procedure were transferred to soil, and continuous watering with vitamin B6 allowed the plants to reach the reproductive stage of development (Fig. 1D and E). Unless the \textit{pdx2} plants (the genotype of which were confirmed by PCR, data not shown) were continuously watered with the vitamin, they became chlorotic and died. A comparison of heterozygous and homozygous \textit{pdx2} plants, which had been deprived of vitamin B6 supplementation for 28 days, demonstrated that the latter had a vitamin B6 and chlorophyll content, reduced 8.6- and 2.2-fold, respectively (Fig. 1F). Nonetheless, the entire seed-set of all rescued/supplemented \textit{pdx2/pdx2} plants were albino (Fig. 1E, inset), demonstrating that vitamin supplementation is not sufficient to support the developing embryo and in turn highlighting the importance of the PDX2 protein in embryo development.

Expression analysis of \textit{PDX2}

The dependence of the \textit{pdx2} mutant on vitamin B6 during development until seed set suggested that \textit{PDX2} is expressed throughout the life cycle. Both a quantitative RT-PCR and immunochemical analysis with a specific antibody, of rosette leaves over an eleven-week period revealed that this is indeed the case (Fig. 2A). \textit{PDX2} appears to be ubiquitously expressed throughout development but there is some variation in the levels observed (Fig.
Levels of transcript abundance were similar in almost all tissues examined, i.e. roots, stems, cauline leaves, rosette leaves, flowers, siliques and cotyledons, with the lowest and highest level being observed in cotyledons and siliques, respectively (Fig. 2B). Tissue levels of the PDX2 protein were positively correlated with the transcript levels, with the notable exception of the abundance of the protein in roots (Fig. 2B). The cause for the diffuse protein band observed for leaf extracts (Fig. 2B) is not known at present. In addition, similar to what has been observed with PDX1 (Titiz et al., 2006), it appears that expression of PDX2 is regulated by light albeit in a slightly different manner. After transfer of etiolated seedlings to light, the level of PDX2 transcript gradually increases up to five hours followed by a decline (Fig. 2C); this decrease is not observed with PDX1 which appears to continuously increase over the same period (Titiz et al., 2006). Light-induced PDX2 transcript levels correlated largely with protein levels, with the exception that the level of PDX2 in etiolated seedlings was much lower than that under long day conditions (Fig. 2C). The latter observation may be indicative of a negative post-transcriptional regulation of PDX2 in the dark.

Glutaminase activity of PDX2

In certain lower organisms PDX2 has been identified as the glutaminase domain of the glutamine amidotransferase, PLP synthase (Belitsky, 2004; Dong et al., 2004; Gengenbacher et al., 2006). Here, we have established functionality of the plant protein by demonstrating glutaminase activity of the isolated recombinant protein (Fig. 3). Activity as a function of the glutamine concentration followed typical Michaelis-Menten kinetics from which the catalytic constants could be estimated ($k_{\text{cat}} = 0.23 \pm 0.01 \text{ min}^{-1}$ and $K_M = 1.92 \pm 0.32 \text{ mM}$) (Fig. S1). While the $K_M$ is within the range of those observed with the orthologous Bacillus subtilis (Raschle et al., 2005) and Plasmodium falciparum (Gengenbacher et al., 2006) enzymes, the $k_{\text{cat}}$ is substantially lower (Table I). Furthermore, in contrast to what has been observed previously with the bacterial and apicomplexan proteins (Raschle et al., 2005; Gengenbacher et al., 2006), we could detect a basal glutaminase activity of the plant PDX2 in the absence of its partner protein PDX1 (0.04 min$^{-1}$); in the presence of PDX1, the PDX2 activity increased approximately 4-fold (Fig. 3A). The level of activity was not appreciably different with either PDX1.1 or PDX1.3, whereas PDX1.2 did not significantly enhance the glutaminase activity of PDX2 (Fig. 3A). Optimal activity was observed when the functional PDX proteins were in a 1:1 molar ratio indicating a stoichiometric protein complex (Fig. 3B).

We noticed that plant PDX2s have a C-terminal extension of approximately 30 amino acids that is not present in the orthologous proteins (Fig. S2A). The cytosolic localization of
PDX2 (Tambasco-Studart et al., 2005) precludes this sequence from a targeting function within the compartmental context of the plant cell. In an attempt to define the function of this domain, we constructed a deletion mutant of PDX2 lacking the C-terminal 30 amino acids (PDX2ΔC30) and determined the kinetic parameters for glutamine hydrolysis. A comparison between the full-length and truncated version of the protein, respectively, shows that the catalytic efficiency ($k_{cat}/K_M$) of the reaction is reduced approximately 2.4-fold in the deletion mutant (Table I) and indicates that the C-terminal amino acids are important for efficient catalysis in Arabidopsis PDX2.

**Biochemical characterization of Arabidopsis PLP synthase**

Previously we reported that Arabidopsis PDX1.1 and PDX1.3 can catalyze PLP formation using either of the pentose phosphate sugars, ribose- or ribulose 5-phosphate, and either of the triose phosphate sugars, glyceraldehyde 3-phosphate or dihydroxyacetone phosphate in the presence of an external ammonium source (Tambasco-Studart et al., 2005). Now, with the availability of a functional recombinant PDX2, we can provide a more detailed analysis of PDX1 activity in the presence of its *in vivo* partner enzyme, PDX2. For an accurate determination of PLP synthase activity, PDX1 was first depleted of the co-purifying pentose sugar (see below) as recently described in (Raschle et al., 2007). With glutamine as the nitrogen source, PLP synthase activity of PDX1 was strictly observed only in the presence of PDX2. Under these conditions, the activity of PDX1 displayed no preference for the triose sugar used but clearly preferred the pentose to the pentulose (Fig. 4A). As our earlier analysis of PLP synthesis using ammonium sulfate as the nitrogen source was biased by the presence of the co-purifying pentose sugar, we reanalyzed PLP synthesis under conditions where no pentose sugar was bound to PDX1. Again there was a preference for the pentose sugar (data not shown), but the rate with dihydroxyacetone phosphate as the triose substrate was significantly decreased. Interestingly, if PDX2 was added, the rate was restored to that observed with glyceraldehyde 3-phosphate (Fig. 4B). The activities observed with either PDX1.1 and PDX1.3 were very similar, whereas no activity was observed with PDX1.2 under any of the conditions used (Fig. 4C), corroborating our earlier analysis (Tambasco-Studart et al., 2005) that PDX1.2 is not functional in PLP biosynthesis.

**Evidence for coupling between PDX1 and PDX2**
The glutaminase and synthase active sites are remote from each other in *Bacillus subtilis* PLP synthase (Strohmeier et al., 2006). Thus, akin to all other characterized glutamine amidotransferases, it is assumed that the ammonia produced at the PDX2 active site travels through a tunnel to the PDX1 active site where it is incorporated into the pyridine ring. In order for this process to work efficiently and to prevent the futile hydrolysis of glutamine, the catalytic activities of the two active sites should be coordinated. We investigated this in both the plant and the bacterial PLP synthase. The fractional ratio for the rate of PLP synthesis versus glutamine hydrolysis can be taken as a measure of channeling efficiency between the two active sites (Bera et al., 2000). Channeling is more pronounced (ca. 7-fold) with the *A. thaliana* proteins than with the *B. subtilis* proteins (Table II). This is predominantly manifested in the lower rate of glutaminase activity of the Arabidopsis proteins (Table II) and implies that the coupling of the activities is stronger between the two plant proteins. Furthermore, we observed that as for the bacterial proteins (Raschle et al., 2005), the substrates of PDX1, either alone or in combination, have no effect on the glutaminase activity of PDX2 (data not shown), indicating that the coupling effect may be inherent to the protein anatomy.

With the elucidation of the structure of the bacterial PLP synthase complex, a proposal was put forward for the route of ammonia transfer between the glutaminase and synthase active sites (Strohmeier et al., 2006; Zein et al., 2006). Specifically, in *B. subtilis* PLP synthase a series of four methionine residues (M13, M145, M79 and M43) that line the putative tunnel have been implicated as having the conformational plasticity to mediate ammonia transfer (Strohmeier et al., 2006). While three of these methionine residues are tightly conserved (M145, M79 and M43 in *BsPdx1*), the one at the interface of Pdx1 and Pdx2 (M13 in *BsPdx1*) is not (see discussion and Fig. 7B). In Arabidopsis PDX2, the equivalent of M13 in *BsPdx1* is replaced by a leucine (L30), a residue that would be expected to display considerable less flexibility. In this context, we were prompted to investigate whether L30 has a role in the enhanced coupling observed with the Arabidopsis proteins compared to the bacterial ones. Indeed, the L30A mutant of PDX1.1 had a substantially reduced coupling efficiency (ca. 4-fold) approaching that observed with the *B. subtilis* proteins; the effects are predominantly manifested by an increase in the rate of glutaminase activity (Table II).
Evidence that the mechanism of action of the plant PLP synthase is similar to that of its prokaryotic counterparts

We have recently identified a covalently bound reaction intermediate during PLP biosynthesis by *B. subtilis*, which is chromophoric, with a characteristic absorbance maximum at 315 nm (Raschle et al., 2007). It is observed with freshly isolated protein and can be reconstituted by addition of the pent(ul)ose phosphate substrate to PDX1 in the presence of PDX2 and glutamine (Raschle et al., 2007). Here we observed that the freshly isolated plant proteins, PDX1.1 and PDX1.3, display an absorbance maximum in this region whereas PDX1.2 does not (Fig. 5A). Furthermore, the chromophoric adduct could be reconstituted with the functional plant PDX1s in a fashion identical to that described for the bacterial protein (Fig. 5B). Formation of the chromophore was absolutely dependent on glutamine hydrolysis by the PDX2 subunit (Fig. 5C). The addition of either of the triose phosphate substrates, glyceraldehyde 3-phosphate or dihydroxyacetone phosphate resulted in its conversion to PLP demonstrating the catalytic competence of the intermediate (Fig. 5D). This data implies that the mechanism of action of PLP synthase is conserved across taxa. We also observed that, similar to the *B. subtilis* protein (Raschle et al., 2007), formation of the chromophore is a function of the concentration of the PDX1 protein (Fig. 6). While PDX2 and glutamine are requisite for chromophore formation by PDX1, only a catalytic amount was necessary, *i.e.* it did not have to be re-added with the subsequent PDX1 additions. This implies that PDX2 is released from the adduct form of PDX1 making it available to activate the newly added free-PDX1.
DISCUSSION

In contrast to what had been tacitly assumed, the de novo pathway of vitamin B6 biosynthesis in plants proceeds via a DXP-independent pathway (Tambasco-Studart et al., 2005). This pathway involves the PDX1 and PDX2 proteins both of which are essential for plant survival as the knockout of either one results in an arrest of embryogenesis at the globular stage (Tambasco-Studart et al., 2005; Titiz et al., 2006). The functionality of PDX2 in the biosynthesis of vitamin B6, both in vivo and in vitro, has been addressed in detail in this study.

The ability to rescue pdx2 seeds by supplementation with vitamin B6 establishes the role of PDX2 in the biosynthesis of this vitamin. Furthermore, the spatial and temporal expression of PDX2 roughly correlates with that observed for the functional PDX1 proteins, PDX1.1 and PDX1.3 (Tambasco-Studart et al., 2005; Titiz et al., 2006), i.e. it is found in all organs examined, is expressed in a similar fashion throughout the developmental cycle, and is light inducible (Titiz et al., 2006). In particular, there is a remarkable similarity at the protein level. At the transcript level, a comparison of their abundance indicates an overall tendency in the order PDX1.3>PDX1.1>PDX2. In some cases PDX2 is more predominant than either PDX1.1 or PDX1.3, e.g. roots and etiolated seedlings (Fig. S3). Data provided by Genevestigator (Zimmermann et al., 2004), support a high correlation between PDX2, PDX1.1 and PDX1.3 but very weak correlation with PDX1.2 (Fig. S4). In contrast to certain other metabolic mutations which can be successfully rescued in a relatively straightforward manner by addition of the missing metabolite, e.g. the biotin defective mutant bio2 (Patton et al., 1998), we did not observe the rescue of mutant pdx2/pdx2 embryos in PDX2/pdx2 plants by providing vitamin B6. Rather, it was necessary to isolate the mutant seeds and continuously supply them with an excess of the vitamin to allow growth. This appears to indicate that there is no transport of vitamin B6 in the silique of Arabidopsis. If the mutant seed is removed from the silique, rescue is possible, implying that uptake can occur through the seed coat. While the homozygous pdx2 plants can be rescued, we have not been able to produce progeny from these plants.

In microorganisms, PDX1 and PDX2 have been clearly proven to function as a glutamine amidotransferase, with both proteins required for the functionality of either domain, i.e. the synthase and glutaminase, respectively (Belitsky, 2004; Dong et al., 2004; Raschle et al., 2005; Gengenbacher et al., 2006). Our data now confirm this for plants as well. The plant and microbial enzymes share properties, e.g. glutaminase activation by PDX1 and dependency on PDX2 for formation of the PDX1 chromophoric adduct; but there are some notable
A key feature of the majority of glutamine amidotransferases is the co-ordination of the glutaminase and synthase activities, thereby preventing the futile hydrolysis of glutamine in the absence of product synthesis. In all glutamine amidotransferases scrutinized thus far, but with the notable exception of PLP synthase (Strohmeier et al., 2006), it appears that coupling of glutaminase and synthase activities is mediated through inter-domain signalling upon binding of the synthase substrate. It has been suggested that a specific inter-domain salt bridge may be responsible for this co-ordination (Myers et al., 2005). As this has not been observed with PLP synthase (Strohmeier et al., 2006), a different mechanism must be anticipated. Formation of an ammonia channel that connects the physically separated active sites provides a mechanism to sequester ammonia from solvent and to deliver it to the PLP synthesis domain for nucleophilic attack on the carbon scaffolds. Specific residues that could control the delivery of ammonia to the PLP synthase active site, in addition to those that control glutaminase activation could be envisaged as additional means to regulate co-ordination between the disparate active sites. As stated above, M13 in *B. subtilis* Pdx1, which is at the base of the predicted ammonia channel, is replaced by a leucine in *A. thaliana* PDX1. Mutation of the latter residue severely disrupts the coupling of glutaminase and synthase activities observed with the plant proteins (Table II). Bacterial PLP synthase appears to only be approximately 15% as efficient as the plant PLP synthase in coupling the respective activities. Indeed, almost 95% of the ammonia derived from glutamine hydrolysis was lost from the bacterial enzyme under our *in vitro* experimental conditions. An additional factor may be required to control coupling *in vivo* with the bacterial system. As a leucine would have different properties than a methionine, the proposed mechanism of entry to the ammonia tunnel must be divergent in the Arabidopsis proteins. To test whether this residue is structurally compatible we created a model of the Arabidopsis PDX1.1 and PDX2 proteins on the basis of the recently solved bacterial PLP synthase complex (Strohmeier et al., 2006). This model indicates that while the core of Arabidopsis PLP synthase would be predicted to be essentially the same as that of *B. subtilis* (Strohmeier et al., 2006) (Fig. 7A), the entrance to the presumed ammonia tunnel must be different (Fig. 7B). This may be the reason for the observation of a less “leaky” tunnel in Arabidopsis.

As observed previously (Tambasco-Studart et al., 2005), PDX1.2 is not able to catalyze PLP biosynthesis *in vitro*, and furthermore, we now show that it is unable to activate the glutaminase activity of PDX2. The structure of the PLP synthase complex has revealed that the N-terminus of PDX1, in particular the unique helix αN, is instrumental not only for
complex formation but also for activation of the glutaminase (Strohmeier et al., 2006). Thus, it is remarkable that despite the extreme conservation of PDX1 across taxa, the most divergent region between the amino acid sequences is in fact the N-terminus (unpublished observation). In particular, key residues involved in the polar contacts that allow precise alignment of $\alpha$N are not conserved in the plant proteins. This is suggestive of a variant mode of interaction between PDX1 and PDX2, and, furthermore, PDX2 activation in Arabidopsis and is supported by the structural model (Fig. 7A). This statement is corroborated further by the fact that *B. subtilis* and *Plasmodium falciparum* PDX1/PDX2 chimeras do not attain full catalytic activity (Gengenbacher et al., 2006). Wagner *et al.* (Wagner et al., 2006) have shown that in contrast to Arabidopsis PDX1.1 and PDX1.3, PDX1.2 does not associate with PDX2. A comparison of the N-termini of the Arabidopsis PDX1s reveals high conservation between PDX1.1 and PDX1.3, while PDX1.2 diverges strongly, and this may explain why it cannot associate with PDX2 (Fig. S2B).

Another notable difference from the bacterial PLP synthase is that efficient utilization of DHAP, but not G3P, as a substrate by Arabidopsis PDX1 is dependent on the presence of PDX2. This indicates that PDX2 induces a conformational change in PDX1 such that it can utilize DHAP with an efficiency equal to that of G3P as the triose sugar in PLP biosynthesis. The exact mechanism behind this observation cannot be deciphered at present, as the binding site of the triose sugar is currently not known. Interestingly, the inability to use DHAP as a substrate in the absence of PDX2 has also been observed for the *P. falciparum* enzyme (Gengenbacher et al., 2006). The *B. subtilis* proteins do not show this dependence, instead Pdx2 is necessary for utilization of the pentulose phosphate sugar, Ru5P, as a substrate (Raschle et al., 2005). This may reflect subtle differences in the active site for the isomerization of triose and pentose sugars and indicates a closer relationship between the plant and apicomplexan machinery, respectively.
MATERIALS AND METHODS

Plant material

Wild type (WT) plants of *Arabidopsis thaliana* (ecotype Col O), the mutant line SALK_072168, as well as the rescued *pdx2* plants were grown on soil at 22°C under 16 hours light/8 hours dark cycles, with 100 µmol photons m⁻² s⁻¹. For the expression analyses, tissue from roots, stems, flowers, siliques, rosette leaves and cauline leaves were collected from at least ten plants. Cotyledons were collected 18 days after stratification 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 kept in the conditions as described above for soil grown plants. For light induction analysis, plates were first 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 transfer to light.

Rescue of *pdx2* seeds

Immature *pdx2* seeds, which have an albino phenotype and are arrested at the globular stage of embryo development (Tambasco-Studart et al., 2005), were isolated from *PDX2/pdx2* plants and placed in an enriched-vitamin medium, composed of MS salts, 3% sucrose, 100 mg/L *myo*-inositol, 500 mg/L 2-(N-morpholino) ethane sulfonic acid (MES), 0.9% agar, 0.1 mg/L 1-naphthylacetic acid, 1 mg/L 6-benzylaminopurine, 1 mg/L biotin, 1 mg/L nicotinic acid, and 1 mg/L thiamine, adjusted to pH 5.7 with KOH. As a control, WT or *PDX2/pdx2* immature seeds, which are green and are not arrested in embryo development, were cultured under the same conditions. Immature seeds cultured in the presence of vitamin B6 were on medium supplemented with 50 µM each of pyridoxol and pyridoxal. The immature seeds were placed on solid medium with additional bathing in liquid medium (composition as above) immediately after their isolation. This liquid medium was supplied at 3-day intervals over 21 days. Growth conditions were 22°C, 16 hours light/8 hours dark and a light intensity of 35 µmol photons m⁻² s⁻¹. The teratomata rescued in the presence of vitamin B6 were transferred to soil after 50 days of growth on the enriched-vitamin medium and were watered daily with a solution of 1 mM pyridoxol and 1 mM pyridoxal.
**Vitamin B6 quantification**

The total amount of B6 vitamers in the rescued *pdx2* and WT (control) plants was estimated by employing a microbiological assay as described by (Tambasco-Studart et al., 2005). Leaves (5 mg fresh weight) were harvested from the rescued plants that had been maintained without vitamin B6 supplementation for 28 days. The vitamin was extracted using 0.02 M H₂SO₄ as described by (Kall, 2003) with the following modifications: a mortar and pestle were used to homogenise the leaf material, the extract was adjusted to pH 5.2 with potassium hydroxide, centrifuged and the supernatant analyzed. Tissue from at least three independent rescued plants was used.

**Measurement of chlorophyll content**

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

**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), 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 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. Quantitative 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 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 ∆Ct method and normalized against the constitutively expressed actin-2 (At3g18780) or ubiquitin (At5g25760) gene. The primers used were: PDX2 forward, 5´-AGATGGGGAAACCTGTTTGG-3´; PDX2 reverse, 5´-CTGTATGTCTCTGCCCACC-3´; actin-2 forward, 5´-ATTCTTGCTTTCCCTCAGCAC-3´; actin-2 reverse, 5´-CCCCAGCTTTTTAAGCCTTT-3´. The control primers for the Arabidopsis ubiquitin gene were from Sigma-Aldrich (Buchs, Switzerland).
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 (Sigma-Aldrich). 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 subjected to 12.5% SDS-polyacrylamide gel electrophoresis (30 µg per lane). Immunodetection was carried out essentially as described by (Titiz et al., 2006), with the exception that the primary antibody used was that raised against the recombinant Arabidopsis PDX2 in rabbit (1:1000 dilution).

Cloning and expression of PDX2

PDX2 was amplified by PCR from isolated cDNA with the primers 5´-GGAATTCCATATGACCGTCGGAGTTTTAGCTTTGC-3´ (forward) and either 5´-CCGCTCGAGTTGAAATATAGGAAGATCAGGCTTAG-3´ or 5´-CCGCTCGAGGCTCCTTGCTCAATCTCTTTCGTCATC-3´ (reverse), to obtain sequences encoding the full-length protein and a truncated version lacking the terminal thirty amino acids, respectively. In both cases, the gene was cloned without the stop codon into the NdeI/XhoI restriction sites of pET21a (Novagen), such that the proteins were expressed with a C-terminal hexa-histidine affinity tag. The constructs, pET-AtPDX2H and pET-AtPDX2ΔC30H were verified by sequence analysis after transformation into DH5α cells (Stratagene) and were transformed into E. coli BL21 cells for expression. Expression of the proteins was induced by addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.25 mM and growth at 26°C for 6 hours. The cells were then harvested by centrifugation and stored at -80°C until further purification.

Protein extraction and purification

In the case of PDX2 and PDX2ΔC30, cell paste from 2 liters of culture was resuspended in 25 ml of lysis buffer (50 mM phosphate buffer pH 8.0, containing 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 2 mM PMSF and 10 mM imidazole). Lysozyme (1 mg/ml) was added and after 30 minutes on ice, the suspension was further lysed by sonication followed by centrifugation at 25,000 g for 30 min at 4°C. The extracted protein was subjected to nickel-
nitrilotriacetic acid chromatography according to the protocol supplied by the manufacturer (Qiagen). Isolated protein was promptly desalted using a PD10 column (BioRad) into storage buffer (20 mM Tris-Cl pH 8.0, containing 10 mM NaCl and 0.1 mM EDTA). The purity of the isolated proteins was judged by SDS-PAGE and the identity confirmed by N-terminal sequencing. PDX1.1-1.3 were isolated in a fashion identical to that described previously (Tambasco-Studart et al., 2005).

**Determination of enzyme activities**

Glutaminase and PLP synthase activities of PDX2, and PDX1, respectively, were performed essentially as described by (Raschle et al., 2005) with the exception that 2.5 U of glutamate dehydrogenase was used in the glutaminase assay.

**Coupling efficiency**

To calculate the coupling efficiency between the PLP synthase subunits, the ratio between the rate of PLP synthesis and glutamine hydrolysis was determined. Reactions were carried out in the presence of R5P (0.5 mM), DL-G3P (1 mM) and L-glutamine (10 mM) with 10 µM each of PDX1.1 and PDX2. Rates of glutamine hydrolysis and PLP synthesis were monitored separately as described above.
ACKNOWLEDGMENTS

We are grateful to the SALK institute for supplying line 072168 for analysis.
SUPPLEMENTAL MATERIAL

Supplemental figure 1. Rate of glutamine hydrolysis as a function of the concentration of glutamine. PDX1.1 (10 µM) was incubated with an equimolar concentration of either, full length PDX2 (filled circles), or a C-terminally truncated version of PDX2, PDX2∆C30, (open circles) while systematically varying the concentration of glutamine (0-20 mM). The rate of glutamine hydrolysis was measured employing a coupled assay as described under methods. The data shown is the average of three independent experiments fitted according to the Michaelis-Menten equation \( f(x)=\frac{ax}{b+c} \).

Supplemental figure 2. Sequence analysis of PDX proteins. A, Alignment of PDX2 amino acid sequences from *Arabidopsis thaliana* (At), *Nicotiana tabacum* (Nc), *Oryza sativa* (Os), *Bacillus subtilis* (Bs), *Saccharomyces cerevisiae* (Sc) and *Plasmodium falciparum* (Pf). The blue box highlights the C-terminal extension of the plant PDX2 sequences. B, Comparison of the PDX1 N-termini from *Arabidopsis thaliana*.

Supplemental figure 3. Absolute transcript abundance of *PDX2* (black bars) compared to *PDX1.1* (light gray bars) and *PDX1.3* (dark gray bars). The examples shown are roots and rosette leaves of seedlings 2 weeks after germination under the conditions described for figure 2, and seedlings grown in continuous dark (DD) for 5 days after imbibition. For absolute amounts of mRNA, standard curves were generated using known concentrations of plasmids carrying the *PDX* genes (*PDX2, PDX1.1, PDX1.3*, respectively). Plasmid DNA was diluted to 1 ng µl\(^{-1}\) followed by 10-fold serial dilutions to 1 fg µl\(^{-1}\).

Supplemental figure 4. Gene Correlator analysis of the expression of *PDX2* in relation to *PDX1* (Zimmermann et al., 2004). The plots display *PDX2* and each of the *PDX1* genes (*PDX1.1, PDX1.2* and *PDX1.3*) on the X and Y axes, respectively. Each spot represents one individual GeneChip; red indicates when both *PDX1* and *PDX2* have a detectable level of expression (p-values < 0.05); green indicates when expression of both *PDX1* and *PDX2* is not detected (p-values ≥ 0.05); dark blue, indicates that only *PDX2* (X axis) is present; and light blue, indicates when *PDX1* (Y axis) is present but *PDX2* is absent.
LITERATURE CITED


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


FIGURE LEGENDS

**Figure 1.** Rescue of the homozygous pdx2 mutant by vitamin B6. **A**, an immature silique from a heterozygous PDX2/pdx2 plant, the arrows indicate albino pdx2 seeds. **B**, immature albino seeds isolated from PDX2/pdx2 plants and cultured in the presence (right) and absence (left) of vitamin B6; pictures were taken 14 days after 16 hour day/8 hour night cycles with a light intensity of 35 µmol photons m\(^{-2}\) s\(^{-1}\); a single wild-type seed was included on the – B6 plate as a control (arrow). **C**, samples similar to **B** in the presence of vitamin B6, but under a higher magnification to emphasize the heterogeneous growth between pdx2 teratomata. **D** and **E** show the development of rescued pdx2 plants 15 and 28 days, respectively, after transfer of teratomata to soil; vitamin B6 supplementation was maintained during this time. The insets in **E** show dissected siliques from a rescued pdx2 plant in which all seeds display an albino phenotype and are later aborted. **F**, the top panel shows the chlorophyll content of PDX2/pdx2 plants (black bars) and rescued pdx2 plants (gray bars) cultured under the same conditions; the bottom panel shows the vitamin B6 content of the same plants. The measurements were performed 28 days after discontinuing supplementation with vitamin B6.

**Figure 2.** Expression analysis of PDX2. The upper panel shows transcript abundance of PDX2: **A**, rosette leaves of two-, four-, six-, eight- and eleven-week old plants. The asterisk indicates senescent leaves of eleven-week old plants; **B**, roots, stems, cauline leaves, rosette leaves, flowers, siliques and cotyledons; **C**, five-day old etiolated seedlings fifteen minutes, one hour, five hours and twenty four hours after transfer to light, as well as long-day (16 hours of light/8 hours dark) (LD) and continuous dark (DD) controls. All values are presented as pg cDNA/µg total RNA. For absolute amounts of mRNA, standard curves were generated using known concentrations of a plasmid carrying the PDX2 gene. Plasmid DNA was diluted to 1 ng µl\(^{-1}\) followed by 10-fold serial dilutions to 1 fg µl\(^{-1}\). The results shown are the average of the experiment performed in triplicate. The lower panel shows PDX2 protein abundance as assessed by an immunochemical analysis. Total protein was extracted from the same samples as described for the upper panel and probed with an antibody raised and purified against the recombinant PDX2. Thirty µg of total protein was loaded per lane. Molecular mass standards are as indicated.

**Figure 3.** Glutaminase activity of PDX2. **A**, initial rates of PDX2 glutaminase activity either in the absence of PDX1 (dashed and dotted line) or in the presence of PDX1.1 (solid line),
PDX1.2 (dotted line), or PDX1.3 (dashed line). In every case 10 µM of each protein was used and 20 mM glutamine.  

B, rate of PDX2 (10 µM) glutaminase activity as a function of the concentration of PDX1.1 (molar ratio 0 - 3.0).

**Figure 4.** Enzymatic formation of pyridoxal 5'-phosphate.  

A, initial rates of activity observed in the presence of ribose 5-phosphate (0.5 mM) and either DL-glyceraldehyde 3-phosphate (1 mM) or dihydroxyacetone phosphate (0.5 mM) (solid and dashed line, respectively), or ribulose 5-phosphate (0.5 mM) and either DL-glyceraldehyde 3-phosphate (1 mM) or dihydroxyacetone phosphate (0.5 mM) (dotted and dashed/dotted line, respectively). All assays shown were carried out in the presence of 20 mM glutamine and 10 µM each of PDX1.1 and PDX2.  

B, initial rates observed as a function of the triose sugar in the presence of ammonium sulfate as the nitrogen source: ribose 5-phosphate and DL-glyceraldehyde 3-phosphate in the presence or absence of PDX2 (solid and dashed line, respectively) or ribose 5-phosphate and dihydroxyacetone phosphate (dotted and dashed/dotted line, respectively). PDX1.1 was present under all conditions shown.  

C, comparison of PLP synthase activity employing all three PDX1 homologs in the presence of PDX2; PDX1.1 (solid line), PDX1.2 (dotted line) and PDX1.3 (dashed line). The assays were performed using ribose 5-phosphate, DL-glyceraldehyde 3-phosphate and glutamine. The concentrations of each component in B and C were as described in A.

**Figure 5.** Characteristics of the chromophoric reaction intermediate.  

A, UV-Visible absorbance spectra of freshly isolated PDX1.1, PDX1.2 and PDX1.3, respectively, in 50 mM Tris-Cl, pH 8.0.  

B, reconstitution of the observed chromophoric adduct employing free-PDX1.1 (20 µM), PDX2 (20 µM), glutamine (10 mM) and ribose 5-phosphate (0.2 mM) in Tris-Cl pH 8.0. The pentose sugar was added at time 0 and the spectra were acquired after 1.5, 4.5, 6.0, 7.5, 9.0, 12.0, 25.5 and 27 minutes.  

C, formation of the chromophoric adduct in the presence (solid line) and absence (dashed line) of glutamine (10 mM) employing PDX1.1 (10µM), PDX2 (10µM) and ribose 5-phosphate (0.3 mM) in Tris buffer pH 8.0.  

D, demonstration of the catalytic competence of the chromophoric adduct; after reconstitution of the latter as described in B (taken as time 0), DL-G3P (0.3 mM) was added and spectra were acquired at 3, 6, 9, 12, 15, 18, 19.5 and 21 minutes. The direction of the arrows indicates either the decrease or increase in absorbance observed.
**Figure 6.** Formation of the chromophoric adduct as a function of the concentration of PDX1. PDX1.1 was added at the time intervals indicated by arrows (10µM at each time point) to a preincubation mix of PDX2 (10µM), glutamine (10 mM) and ribose 5-phosphate (0.3 mM) in 50 mM Tris-Cl pH 8.0.

**Figure 7.** Modeling the 3D structure of the domains of PLP synthase from *Arabidopsis thaliana*. A, a ribbon representation of PLP synthase from *Bacillus subtilis* (PDB code 2NV2) is shown in green and is overlaid by a model of PDX1.1 (gold) and PDX2 (red) from Arabidopsis. The two figures show views turned by 90° around a vertical axis in the paper plane. Sequence insertions in the plant enzymes compared to the bacterial enzyme have not been modelled, but are indicated in the figure. Visible secondary structure elements have been labelled for reference. B, the part of PLP synthase depicted is proposed to be the route of ammonia transfer. Residues involved in the *Bacillus subtilis* protein are shown in ball and stick representation (green) overlaid by the equivalent residues of Arabidopsis PDX1.1 (gold). A number of residues are seen to be different in PDX1.1, notably M13 in *Bs*Pdx1 is replaced by a leucine.
Table I. Steady state kinetic constants of PDX2

Kinetic parameters were determined as a function of the glutamine concentration by measuring the PDX2 glutaminase activity using a coupled assay (Raschle et al., 2005). In the case of Arabidopsis thaliana, 10 µM each of PDX1 and PDX2 were used.

<table>
<thead>
<tr>
<th>Species</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (mM min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Bacillus subtilis</td>
<td>7.6</td>
<td>0.99</td>
<td>7.68</td>
</tr>
<tr>
<td>† Plasmodium falciparum</td>
<td>6.6</td>
<td>0.56</td>
<td>11.8</td>
</tr>
<tr>
<td>Arabidopsis thaliana (full length)</td>
<td>0.23 ± 0.01</td>
<td>1.92 ± 0.32</td>
<td>0.12</td>
</tr>
<tr>
<td>Arabidopsis thaliana (ΔC30)</td>
<td>0.13 ± 0.01</td>
<td>2.62 ± 0.35</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Taken from (Raschle et al., 2005)
† Taken from (Gengenbacher et al., 2006)
Table II. Coupling efficiency of PLP synthase

PLP formation and glutamine hydrolysis were measured and the ratio between the two was used to estimate the coupling efficiency between PDX1 and PDX2.

<table>
<thead>
<tr>
<th>Species</th>
<th>(^a\text{PLP} \quad (\text{min}^{-1}))</th>
<th>(^b\text{Glutaminase} \quad (\text{min}^{-1}))</th>
<th>(^b\text{Coupling})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana PDX1.1 WT</td>
<td>0.13 ± 0.008</td>
<td>0.37 ± 0.050</td>
<td>0.35</td>
</tr>
<tr>
<td>Arabidopsis thaliana PDX1.1 L30A</td>
<td>0.11 ± 0.020</td>
<td>1.14 ± 0.020</td>
<td>0.09</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.15 ± 0.006</td>
<td>3.43 ± 0.190</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^a\) Employing 10 \(\mu\)M each of PDX1 and PDX2 with R5P and DL-G3P as substrates

\(^b\) Ratio between the respective reaction rates of PLP synthesis and glutaminase
Figure 3

A

![Graph showing absorbance at 363nm vs. time (min) for different molar ratios of PDX1.1 and PDX2.]

B

![Graph showing observed rate constant ($k_{obs}$) vs. PDX1.1 / PDX2 molar ratio.]

Figure 6

Absorbance at 315 nm

Time (min)
Scheme 1

Glutamine → Glutamate

PDX2

-2O3PO
\[\text{NH}_3\]

Ribose 5-phosphate

\[\text{HO} \quad \text{OH} \quad \text{HO} \quad \text{OH}\]

-2O3PO

PDX1

\[\text{HO} \quad \text{OH} \quad \text{OH}\]

Dihydroxyacetone phosphate

-2O3PO

Ribulose 5-phosphate

\[\text{HO} \quad \text{OH} \quad \text{OH}\]

Pyridoxal 5'-phosphate