PYRIDOXINE (PYRIDOXAMINE) 5'-PHOSPHATE OXIDASE

IN *ARABIDOPSIS THALIANA*

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PYRIDOXINE (PYRIDOXAMINE) 5'-PHOSPHATE OXIDASE IN *ARABIDOPSIS THALIANA*

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
December 19, 2008

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Yuying Sang

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Yuying Sang, daughter of Shiqing Sang and Guilan Wang, was born on January 7, 1975, in Chiping, Shandong, People's Republic of China. She received the Bachelor of Science degree in Biology in July 1997 from Shandong Normal University and entered the Graduate School of Kunming Institute of Botany, Chinese Academy of Sciences. In the July of 2000, she graduated with a Master of Science degree in Botany and joined East China University of Science and Technology as a lab manager in the Department of Bioengineering. In August 2003, she enrolled in Auburn University to pursue a Doctor of Philosophy Degree in the Department of Biological Sciences. She married Daike Tian in 2000 and had their first child, Olivia Tian, in 2006.

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Doctor of Philosophy, December 19, 2008
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106 Typed Pages

Directed by Narendra K. Singh

Vitamin B_6 is the collective term for a group of three related compounds, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated derivatives, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). Many enzymes are involved in the metabolism of vitamin B6, one of which is pyridoxine (pyridoxamine) 5'-phosphate oxidase (PPOX). PPOX has been well studied from bacteria, fungi and animals, but little research about PPOX has been done in plants.

In this study, two genes in the genome of *Arabidopsis thaliana* were found to encode for proteins that demonstrated PPOX activity: one gene is *At5g49970* coding for a protein now designated AtPPOX-1, and the other gene is *At2g46580* coding for a protein now designated AtPPOX-2. The cDNAs of *At5g49970* and *At2g46580* were cloned using

the RT-PCR method, and then subcloned into the *E. coli* expression vector and subsequentely into yeast shuttle vector. PPOX functionally was demonstrated in two ways, i.e. demonstration of PPOX enzyme assay directly *in vitro* and by the complementation of a PDX3 (coding for yeast PPOX) knockout of yeast for increased oxidative stress susceptibility. However, the enzyme activity of AtPPOX-1 is almost 300-fold higher than that of AtPPOX-2. The inferred amino acid sequence of AtPPOX-1 is 530 amino acids long and that of AtPPOX-2 is 198 amino acids.

Although AtPPOX-1 and AtPPOX-2 are putatively isoenzymes, very little homology was found in their amino acid sequences. In order to understand their evolutionary relationship, phylogenetic analysis of AtPPOX-1 and AtPPOX-2 homologs across the three domains of life suggests that AtPPOX-1 and AtPPOX-2 have independent origins. AtPPOX-1 phylogeny appears congruent with the underlying branching pattern of the overall tree of life, while AtPPOX-2 phylogeny suggests that plant PPOX-2 may have originated from cyanobacteria. Presence of Yjef_N domain in land plants AtPPOX-1 homologs suggests that acquisition of this domain and its fusion with pyridox_oxidase domain began with the endosymbiotic acquisition of the chloroplast.

In this study, regulation of the *AtPPOX-1* gene and its *in vivo* physiological functions were investigated. Variable levels of expression of *AtPPOX-1* were seen in all tissues of *A. thaliana* examined. This gene is up-regulated by light, heat shock, ABA, JA and ethylene treatment, and down-regulated by exposure to NaCl. *AtPPOX-1* may have two alternative splicing variants. One is expressed in all tissues, but the other is not expressed in root. To determine the biological role of *AtPPOX-1*, T-DNA insertional

mutants of *A. thaliana* in *AtPPOX-1* were analyzed. The mutant lines showed sensitivity to NaCl and high light for growth, and sensitivity to high concentrations of sucrose in cotyledon development. These results suggest involvement of *AtPPOX-1* in stress tolerance and cotyledon development in *A. thalia*

ACKNOWLEDGEMENTS

I would like to express my gratitude to Drs. Narendra Singh, Robert Locy, Joe Cherry, Joanna Wysocka-Diller, and Fenny Dane for their guidance and assistance during my graduate studies. My major advisor, Dr. Singh, impressed me in many ways. Not only did he teach me how to do research, but he also taught me how to be a person. He is the nicest person I have ever met. Dr. Locy's full of knowledge and ideas, always provided me with good suggestions whenever I met problems in the research. I am also grateful for the help from Dr. Leslie Goertzen for his assistance with the phylogenetic analysis. Thanks are due to Dr. Roland Dute who instructed me on how to do immunohistochemistry and allowed me to work in his lab, and also to Dr. Michael Miller who mentored me in immunocytochemisty. As a lab technician and my friend, Ms. Chia-chen Weng gave me much support that assured my experiments moved on smoothly. My labmate, Dr. Nisreen AL-Quareen, also gave me help during my studies. I would like to give many thanks to all other persons in the Biology Department in Auburn University.

My parents, Shiqing Sang and Guilan Wang, my sisters, and my husband, Daike Tian, gave me great support throughout my graduate studies. I can not finish my degree without their selfless help.

Style manual or journal used: Plant Physiology

Computer software used: Microsoft Word 2003

Microsoft Excel 2003

Sigma Plot 2000

Adobe Photoshop

Paup

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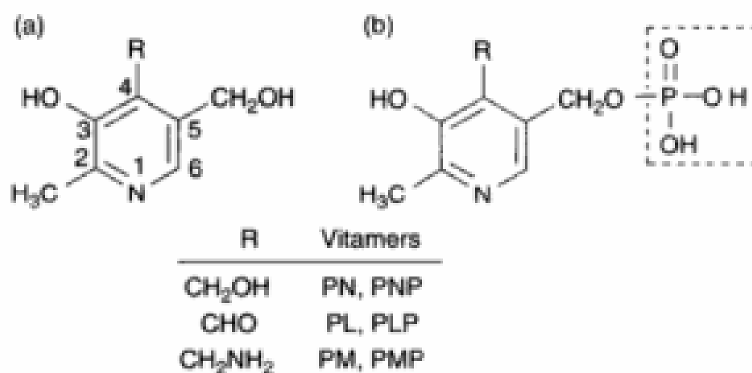
I LITERATURE REVIEW

VITAMIN B_6

Vitamins are organic compounds required in tiny amounts for essential metabolic reactions in living organisms (Lieberman, 1990). Human need 13 vitamins of which 4 (A, D, E and K) are fat-soluble and 9 (8 B vitamins and vitamin C) are water-soluble.

Vitamin B_6 is a collective term used to describe pyridoxal, pyridoxine, pyridoxamine and their respective 5´-phosphorylated forms: pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP) (http://www.chem.qmul.ac.uk /iupac/misc/B6.html). The chemical entity present at the 4' position of the pyridine ring defines the different vitamers, which can be an aldehyde (pyridoxal), an alcohol (pyridoxine) or an amine (pyridoxamine). Phosphorylation of 5'-position forms their corresponding phosphorylated forms as shown below.



A ubiquitous bound form of PN, 5'-O-(-D-glucopyranosyl)-pyridoxine, abbreviated as PN-glucoside is found in many plant tissues. It has been reported that 60-90%

of the B_6 vitamers contained in certain foods are in the form of PN-glucoside (Gregory and Nakano, 1997; Gregory and Sartain, 1991). PN-glucoside partially impairs the metabolic utilization of co-ingested non-glycosylated forms of vitamin B_6 (Gilbert and Gregory 1992), because it uses the same transport system as pyridoxine (Zhang and Mccormick, 1992). Upon entry into the cell, PN-glucoside undergoes hydrolysis to release pyridoxine, which is the rate limiting step in metabolism of this β-glucoside (Zhang and Mccormick, 1993).

FUNCTIONS OF VITAMIN B_6

Vitamin B_6 is an essential metabolite for all organisms and plays important role in the regulation of many metabolic activities in the cell. Vitamin B6 is well known for its role as cofactor of many enzymes. PLP-dependent enzymes catalyze over 100 enzymatic reactions, predominantly in amino acid metabolism including transamination, decarboxylation, racemization, Cα-Cβ bond cleavage and α, β- elimination reactions (Eliot et al., 2004; Drewke et al., 2001; John R, 1995; Fitzpatrick et al., 2007). In its role as a coenzyme, PLP is bound tightly to the apoenzyme by a Schiff base (aldimine) linkage formed through condensation of the 4-carbonyl group with the ε-amino group of specific lysine residues. The resultant Schiff base compound may be subject to nucleophilic attack by a neighboring amino, sulfydryl, or imidazole group to form a substituted aldamine (Ball GF, 2006). All vitamers of vitamin B_6 are reversibly bound and easily dissociate from the apoenzymes.

Recent interest in vitamin B_6 study has increased because of its potential antioxidant properties. While studying mechanisms of resistance of *Cercospora nicotianae* to singlet-

oxygen-generating phytotoxins, it was found that the resistance was dependent upon the expression of a gene essential for synthesis of vitamin B_6 (Ehrenshaft et al., 1999). Comparable to vitamin C and E, vitamin B_6 and its derivatives are efficient quenchers of singlet oxygen ($^1O_2$), a strong oxidizer that initiates radical oxidation in biological systems (Bilski et al., 2000). Vitamin B_6 and its derivatives are converted to endoperoxide and hydroperoxide intermediates in the reaction of vitamin B_6 with $^1O_2$ (Ohta and Foote, 2002). Vitamin B_6 has been linked to oxidative stress in diverse organisms. Antioxidant effect of vitamin B_6 on *Schizosaccharomyces pombe* cells treated with menadione is due to reduction of glutathione content. This reduction in turn suppressed an increase in peroxide and thiobarbituric acid reactive substances, and increased the viability of the yeast cells under oxidative stress (Chumnantana et al., 2005). Pyridoxine/pyridoxamine 5'-phosphate oxidase knockout mutant in *Saccharamyces cerevisiae* is sensitive to H_2O_2 stress (Sang et al, 2007). Involvement of vitamin B6 has been shown in oxidative stress abatement. Mechanisms underlying its function in this complex process require more investigation (Jain and Lim, 2001).

Vitamin B_6 may function as a regulator of a number of membrane ion transporters. The enhanced influx of calcium ions into an intracellular compartment of the vascular smooth muscle resulting in hypertension in rat could be blocked by PLP and by dihydropyridine-sensitive calcium channel blockers (DHP), suggesting that PLP could be an endogenous modulator of DHP – sensitive calcium channels (Dakshinamurti et al., 1998). In addition, vitamin B_6 has been linked to modulation of hormone function because of its ability to bind to steroid receptors (Oka T, 2001), and through modulation

of transcription factors by PLP conjugation to transcriptional coregulators (Mostaqul et al., 2007).

Because of the essential functions of vitamin B_6 in all organisms, many efforts have been directed into its potential use in curing some diseases in humans. There is growing evidence that vitamin B_6 suppresses colon tumorigenesis by reducing cell proliferation, oxidative stress, NO production and angiogenesis (Komatsu et al., 2003). PLP and PL act as a lipid glycation inhibitors, which presumably contributes to diabetes prevention (Higuchi et al., 2006). Type 2 diabetic patients have an abnormal increase of phosphatidylcholine hydroperoxide (PCOOH) (Nagashima et al., 2002). PCOOH may be related to Amadori-glycated phosphatidylethanolamine (Amadori-PE) which generates reactive oxygen species and thereby triggers lipid peroxidation (Oak et al., 2000). PLP and PL could easily be condensed with PE before the glucose/PE reaction occurred (Higuchi et al., 2006). Advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) are implicated in many age-related chronic diseases and in protein aging. PM is not only an inhibitor of AGEs, but also inhibits ALEs by trapping malondialdehyde (MDA) directly under physiological conditions, and provides insight into the mechanism of action of PM in protecting proteins against carbonyl stress (Onorato et al., 2000; Kang et al., 2006).

PLP has also been implicated as a precursor of vitamin B_1 (thiamine) biosynthesis in yeast (Zeidler et al., 2002). The pyrimidine unit of thiamin (Vitamin B1) originates from a C5N fragment, derived from C-2',2,N,C-6,5,5' of pyridoxol (Vitamin B_6) and an N-C-N fragment derived from L-histidine. Urocanic acid serves as an intermediate in the

insertion of the N-C-N fragment of histidine into the thiamin pyrimidine (Zeidler et al., 2003).

PLP is synthesized in plants using the DXP-independent biosynthesis pathway in which Pdx1 and Pdx2 proteins are involved (Studart et al., 2005). Genes encoding PDX1 have been identified in several plant species, and their functions are extensively studied (Shi et al., 2002; Chen and Xiong, 2005; Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Studart et al., 2007). *PDX1* is required for shoot and root development. *Arabidopsis pdx1* knockout mutants were impaired in root growth and early seedling development (Chen and Xiong, 2005). In addition to the developmental defects, *PDX1* mutants are hypersensitive to salt, osmotic stress and oxidative stress (Chen and Xiong, 2005). These mutant seedlings had increased peroxidation of membrane lipids following UV treatment. These studies establish a critical role of vitamin B6 in plant development and stress tolerance and suggest that vitamin B_6 may represent a new class of antioxidant in plants (Chen and Xiong, 2005; Titiz et al., 2006;Wagner, et al., 2006). No homozygous plants could be obtained for *pdx2* T-DNA insertion mutants, suggesting that *PDX2* is involved in the seed development (Studart et al., 2005). *Pdx1* and *pdx2* are regulated by stressors, such as high light, chilling, drought, ozone and plant pathogen, which cause oxidative stress indirectly (Denslow et al., 2005; Denslow et al., 2007). These results support a role for vitamin B_6 as an antioxidant and modulator of active oxygen species *in vivo*.

Pyridoxal kinase catalyzes the phosphorylation of the vitamin B_6 vitamers to form the corresponding phosphorylated derivatives (Lum et al., 2002). It is involved in the salvage pathway of the vitamin B_6 biosynthesis. Root growth of *A. thaliana SOS4* mutant

encoding pyridoxal kinase is slower than that of the wild type, and the mutants do not have root hairs in the maturation zone, suggesting that pyridoxal kinase is required for root hair development in *Arabidopsis* (Shi and Zhu 2002). But how the pyridoxal kinase affects the root hair development was not explained. Shi et al. (2002) have also demonstrated that pyridoxal kinase is a salt tolerance determinant important for the regulation of Na^+ and K^+ homeostasis in plants, and proposed that PLP regulates Na^+ and K^+ homeostasis by modulating the activities of ion transporters.

Pyridoxamine (pyridoxine) 5'-phosphate oxidase (PPOX), converting PMP and/or PNP to PLP, is another enzyme involved in the salvage pathway. Loss of PPOX activity seriously disrupts cellular metabolism in several ways, with particular effects on amino acid metabolism. In *E. coli*, excretion of L-glutamate and possibly α- ketoisovalerate, which triggers L-valine inhibition, were shown (Lam and Winkler, 1992). In *Saccharomyces cerevisiae*, pleiotropic phenotypes induced by lack of PPOX activity include strong perturbations in amino acid biosynthesis, atypic fatty acid, sterol and cytochrome patterns, and sensitivity to H_2O_2 stress (Loubbardi et al., 1995). The PPOX mutants of *Arabidopsis* showed decreased growth in root and shoot under normal conditions, and were incapable of increased growth under high light conditions (Gonzalez et al., 2007). However, physiological functions of PPOX in plant growth and development have not been fully determined.

EFFECTS OF DEFICIENCY AND OVERDOSE OF VITAMIN B_6

Humans and animals can not synthesize vitamin B_6 themselves and must obtain it from dietary sources (http://en.wikipedia.org/wiki/vitamin_B6). Either deficiencies or overdoses of vitamin B_6 are known to cause serious problems (Dakshinamurti K., 1990). The classic clinical syndrome for B_6 deficiency is a seborrheic dermatitis-like eruption, atrophic glossitis with ulceration, angular cheilitis, conjunctivitis, intertrigo, and neurologic symptoms of somnolence, confusion, and neuropathy (James et al., 2006; http://en.wikipedia.org/wiki/vitamin_B6). Vitamin B_6 is an important growth factor in rats; a deficiency causes a marked acrodynia type of dertermatitis (Emerson et al., 1938).

An overdose of pyridoxine from vitamin B_6 supplements, although never from food source, can cause a temporary deadening of certain nerves such as the proprioceptory nerves, causing a feeling of disembodiment common with the loss of proprioception (http://dietary-supplements.info.nih.gov/factsheets/vitaminb6.asp). This condition is reversible when vitamin B_6 supplementation is stopped. Very high doses of pyridoxine over long periods of time may result in painful neurological symptoms known as sensory neuropathy (Pfeiffer et al., 1995).

BIOSYNTHETIC PATHWAY OF VITAMIN B_6

Bacteria, fungi, and plants are able to synthesize the vitamin B6 *de novo*, but humans and animals must acquire it through their diet. Two distinctive pathways for its de novo synthesis have been identified to date. These are the deoxyxylulose 5-P (DXP)-dependent pathway and the DXP-independent pathway (Franco et al., 2003; Fitzpatrick et

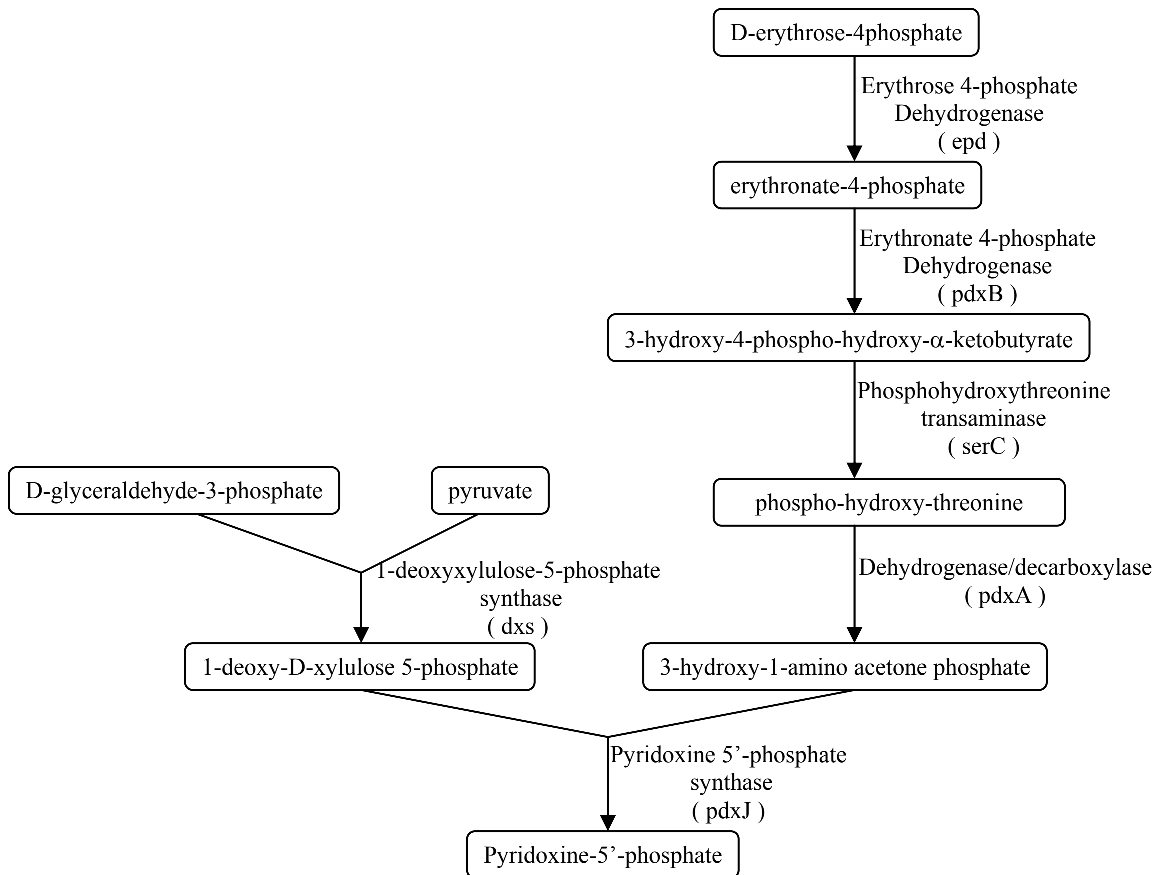
al., 2007). DXP-dependent pathway seems to be restricted to the γ subdivision of protobacteria, while DXP-independent pathway appears to be present in fungi, plants, and other bacteria (Salvo et al., 2003).

Deoxyxylulose 5-P (DXP)-dependent pathway

The vitamin B_6 biosynthesis pathway has been extensively studied in *E. coli* where PNP is derived from 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose-5-phosphate (Laber et al., 1999). It was assumed for a long time that vitamin B_6 is synthesized in the same way in all organisms. However, the deoxyxylulose 5-P (DXP)-dependent pathway has now been shown to be generally limited to the gamma-branch of proteobacteria (Flicker et al., 2005; Mittenbuber 2001)), in which two branches with six enzymatic steps are involved (Fitzpatrick et al., 2007) as shown below in Scheme 1. In one branch, Epd, PdxB and SerC catalyze the conversion of D-erythrose 4-phosphate into phospho-hydroxy-threonine (Lam and Winkler, 1990; Yang et al., 1998) which then undergoes oxidation and decarboxylation by PdxA to form 3-hydroxy-1-aminoacetone phosphate (Banks and Cane, 2004; Cane et al., 1998). In the other branch, DXP is derived from glyceraldehyde 3-phosphate and pyruvate by the action of DXP synthase (Sprenger et al., 1997). The products of the two branches, i.e. 3-hydroxy-1-aminoacetone phosphate and DXP, are then condensed in a reaction catalyzed by PdxJ to form PNP (Laber et al., 1999). PNP is oxidized into the cofactor vitamer PLP by PdxH.

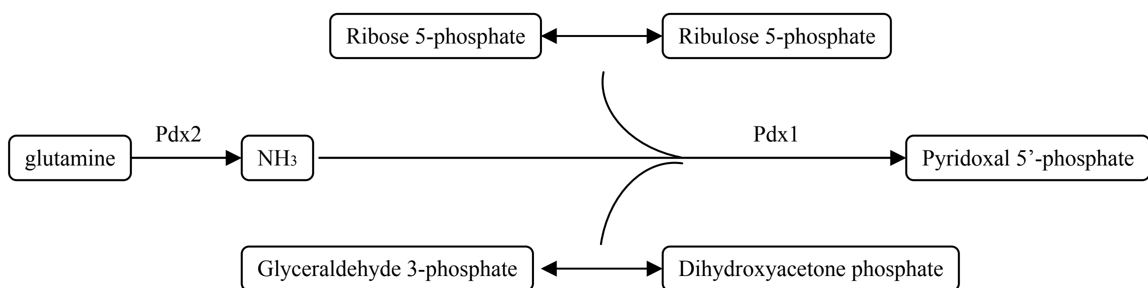
Deoxyxylulose 5-P (DXP)-independent pathway

It has been shown that a large majority of organisms synthesize vitamin B_6 via a completely different pathway from *E. coli* and the rest of the gama-proteobacteia (shown in Scheme 2). In this pathway, PLP is synthesized directly from either ribose 5-phosphate or ribulose 5-phosphate, in combination with either glyceraldehyde 3-phosphate or dihydroxyacetone phosphate, and glutamine as the nitrogen (N) donor (Burns et al., 2005; Raschle et al., 2005). Pdx1 and Pdx2 are the key enzymes involved in the DXP-independent pathway. Neither of these enzymes show similarity to any of those involved in the DXP-dependent pathway (Ehrenshaft et al,. 1999; Ehrenshaft et al., 2001; Studart et al., 2005). Pdx1 protein was found to be one of the most evolutionarily



Scheme 1. Vitamin B_6 biosynthesis by the DXP-dependent pathway.

conserved proteins (Braun et al., 1995). Pdx2 protein is less well conserved but contains several protein motifs that are conserved throughout all Pdx2 proteins (Ehrenshaft et al., 2001). Pdx1 and Pdx2 proteins interact with each other resulting in a functional PLP synthase complex that functions as a glutamine amidotransferase, with the Pdx2 protein as the glutaminase domain and Pdx1 as the acceptor and PLP synthase domain (Belitsky BR, 2004; Dong et al., 2004).

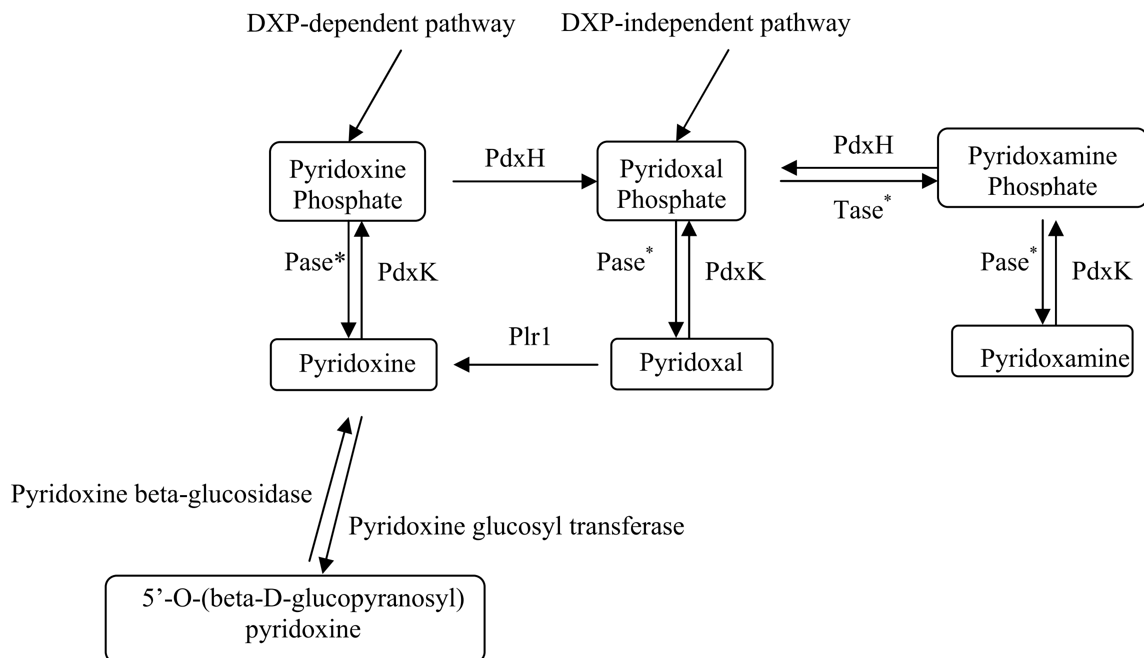


Scheme 2. Vitamin B_6 biosynthesis by the DXP-independent pathway.

Salvage pathway

Most organism possess a vitamin B_6 biosynthesis salvage pathway that interconverts and recycles the various vitamers of pyridoxine. Organisms that do not have the de novo pathway of vitamin B_6 biosynthesis need to acquire vitamin B_6 from their diet, and then interconvert the vitamin B_6 vitamers via this salvage pathway into the active coenzyme (PLP) (Tsuyoshi et al., 2004). Although there is divergence in the *de novo* synthesis pathway of pyridoxine, the salvage pathway appears to be conserved (as shown in scheme 3). In this pathway, pyridoxine, pyridoxal and pyridoxamine can be reversibly phosphorylated and dephosphorylated through the action of specific kinases (PdxK and

PdxY) and apparently unspecific phosphatases respectively (Yang, 1996; Yang, 1998). However, a specific phosphatase has been reported in humans (Fonda, 1992; Jang et al., 2003). Furthermore, PNP or PMP can be converted into PLP by the action of the oxidase PdxH (Zhao and Winkler, 1995), which is included in the *de novo* pathway of vitamin B_6 biosynthesis by the DXP-dependent route. In addition, a pyridoxal reductase has been identified in yeast (Nakano et al., 1999). It is assumed that the majority of organisms have one or the other of these enzymes.



Scheme 3. Vitamin B_6 biosynthesis by the salvage pathway

Evolutionary perspectives of the two independent pathways

The DXP-dependent pathway is restricted to the γ-division of proteobacteria. It has been suggested that, because the γ-division of proteobacteria is thought to represent the

most recent lineage of prokaryotic evolution, the genes of the ancestral *Pdx1/Pdx2* pathway were lost during evolution of the proteobacteria and *PdxA/PdxJ* pathway evolved in this lineage (Mittenbuber, 2001). The reason for the loss of the Pdx1 and Pdx2 genes is unknown. However, there is the hypothesis that there may have been a time during the evolution of the γ-division of proteobacteria where uptake of the vitamin from the environment was selected for and the Pdx1 and Pdx2 genes became dispensable. Then the Pdx1/Pdx2 genes might have been lost without deleterious consequences. Later, it became necessary to produce the vitamin *de novo* leading to 'reinvention' of the synthesis of the pyridoxine ring (Fitzpatrick et al., 2007). Fitzpatrick et al. (2007) also did comparison between the two independent routes to the biosynthesis of vitamin B6. Supprisingly, it was found that the key biosynthetic enzymes of both pathways are, in fact, very similar both structurally and mechanistically.

TRANSPORT OF VITAMIN B_6

Transport of vitamin B6 has been mostly studied in mammals, yeast and bacteria. However, there is no information available about the transport of vitamin B_6 in plants.

Mammals are auxotrophic for vitamin B_6 and must acquire it from their diet. Liver and intestine are very active in the metabolism of B_6 vitamers. In the intestinal lumen, the dietary phosphorylated vitamin B_6 is hydrolyzed into dephosphorylated forms. PL, PM and PN are then absorbed by passive diffusion (Hamm et al. 1979). However, PL is the only form transported in the blood (Sakurai et al., 1991). The absorbed PM and PN are rapidly acted on by PL kinase and then converted to PLP by pyridoxine (pyridoxamine)

5'-phosphate oxidase (PPOX) (Ink SL, 1984). These two enzymes plus phosphohydrolase provide a means of converting dietary PN and PM to circulating PL which is then taken up and phosphorylated into PLP by other organs that contain pyridoxal kinase (Sakurai et al., 1987; Sakurai et al., 1991).

Unlike mammals, yeast is prototrophic for vitamin B_6, but in vitamin B_6 auxotrophic mutants, uptake of PN, PL, or PM from the extracellular space is required to generate intracellular PLP via a salvage pathway. Vitamin B_6 transport has been intensively studied in *S. carlsbergensis*, in which two distinct uptake systems that differ in structural specificity and ionic requirements are present. System 1 has affinity for PL and PN and a pH optimum of 3.5, whereas system 2 is specific for PM and PN and has a pH optimum of 6.0 (Shane and Snell, 1976). For *S. cerevisiae*, Tpn1p was found to be the only functional transport protein for vitamin B6 encoded in the genome. This protein was also shown to mediate the uptake of PL, and to a lesser extent, PM. The activity of Tpn1p is very similar to that of system 1 and the protein that corresponds to system 2 of *S. carlsbergensis* is not known. Moreover, *S. carlsbergensis* contains a protein that is almost identical to Tpn1p and thus is very likely to function in PN uptake (Stolz and Vielreicher, 2003).

No transport protein for vitamin B_6 has ever been found in bacteria. *Salmonella typhimurium* can transport PN and PL with the K_m values of 2.0 x 10^{-7} M and 1.2 x 10^{-7} M, respectively, but this bacteria can not transport pyridoxamine (Mulligan and Snell, 1976). However, a vitamin B_6 auxotroph derived from *E. coli* K12, utilized the three unphosphorylated forms of vitamin B_6, more or less effectively, for growth (Yamada et al., 1977; Mulligan and Snell, 1977).

PYRIDOXINE/PYRIDOXAMINE 5'-PHOSPHATE OXIDASE ACTIVITY

PPOX catalyzes the oxidation of either PNP or PMP into PLP, with FMN as its cofactor and oxygen as the final electron acceptor. During the oxidation, a hydrogen atom is removed from the C4' alcohol group of PNP or amino group of PMP, and a pair of electrons is transferred to tightly bound FMN, forming PLP and $FMNH_2$. PLP contains a very reactive aldehyde group that reacts with virtually all nucleophiles in the cell. How is PLP protected in the cell from adventitious reactions with non-productive nucleophiles? PPOX is a kinetically sluggish but moderately abundant enzyme in *E. coli*. It contains a non-catalytic PLP binding site and a catalytic PLP binding site (Zhao and Winkler, 1995; Yang and Schirch, 2000). PLP dissociates very slowly from the non-catalytic PLP binding site, and this slowly dissociating PLP can be readily transferred to the PLP requiring apoenzyme to form the active holoenzyme. Safo et al. (2001) proposed that a possible tunnel exists between the two sites so that PLP formed at the active sites may transfer to the non-catalytic site without passing through the solvent. However, there is no report about how the non-catalytic binding PLP is transferred to the PLP requiring apoenzymes.

PPOX from different organisms

PPOX was first isolated and purified from rabbit liver (Kazarinoff et. al., 1975). Subsequently, the enzyme was purified from sheep and pig brain (Choi and Churchich, 1987). Wheat seedlings were reported to have at least three PPOX enzymes, but only two

of them were partially purified (Tsuge et. al., 1982). PPOX from *E. coli*, and *A. thaliana* was overexpressed and purified from *E. coli* (Salvo et al., 1998; Sang et al., 2007). Both the prokaryotic and eukaryotic Pyridoxine 5'-phosphate oxidases are homodimers. The two identical monomers are held together via disulfide bonds as well as salt-bridge interactions. Each subunit tightly binds one molecule of PLP and one molecule of FMN as a cofactor (Kazarinoff et al., 1975; Choi et al., 1983; Zhao et al., 1995; Salvo et al., 1998).

Most extensive biochemical studies have been carried out with the PPOX from rabbit liver and *E. coli*., and their kinetic constants for the oxidation reaction catalyzed by PPOX have been determined . PPOX of *E. coli* has the K_m of 2 μM for PNP at 37 °C and pH 8.5 and the K_{cat} of 0.76 s^{-1}. Whereas K_m for PMP is higher around 105 μM and K_{cat} value is 1.72 s^{-1} (Zhao and Winkler, 1995; Salvo, et al., 1998). For the enzyme from rabbit liver, kinetic constants are quite similar for the two substrates (K_m = 8 μM, K_{cat} = 0.7 s^{-1} for PNP; K_m = 6μM, K_{cat} = 0.1 s^{-1} for PMP (Choi et al., 1983). The PMP oxidase activity of the PPOX from *E. coli* showed a sharp pH profile. The PMP oxidase enzyme activity was absent at pH 6, rose sharply as the pH increased from 6 to 8.5, and then remained nearly constant at pH values between 8.5 and 10. In contrast, the PNP oxidase activity showed a very broad pH profile. PNP is by far the better substrate for the PPOX from *E. coli* at pH's above 8.0, whereas PPOX from rabbit liver oxidizes both substrates to almost the same extent. Below pH 8.0, PNP is by far the better substrate for both *E. coli* and rabbit liver PPOX. Because the aminomethyl group of PMP is largely protonated

below pH 9.0, the unprotonated group is attacked preferentially by the enzyme (Wada and Snell, 1961). The PPOX is relatively thermal stable at high pH, and easily loses its activity at lower pH (Zhao and Winkler, 1995; Wada and Snell, 1961).

Mechanistic pathway of the PPOX

The mechanistic pathway of PPOX on a molecular level is: from the resting enzyme with no substrate bound, to the initial binding of PNP at the active site (pre-reaction state), to the catalytic stage and finally to the transfer of the catalytic product PLP from the active site to the PLP binding site for subsequent transfer to vitamin B_6 apoenzyme (Safo et al, 2005). In the absence of PLP, the active site of the enzyme is in an “open” conformation. Once substrate binds and is converted to PLP, the active site of the enzyme is in a partially “closed” conformation. Specific amino acid residues can form hydrogen bonds with the PLP, thus forming a lid that physically covers the active site, giving rise to the “closed” conformation (Yang and Schirch, 2000; Safo et al, 2001). Once the PLP is transferred to the binding site, the active site is open and ready for the next substrate.

Regulation of PPOX

Substrate inhibition was observed using PNP as substrate, with a K_i of 50 μM, and was partially relieved by increasing O_2 concentration (Choi et al., 1983). Substrate inhibition seemed to occur by binding of PNP to the reduced form of the enzyme, forming an abortive complex. On the other hand, no substrate inhibition was observed for PMP oxidase activity (Zhao and Winkler, 1995). PLP is a very strong competitive inhibitor of the oxidase in both *E. coli* and mammals. PLP and PLP analogs were shown

to inactivate PPOX by binding to a specific lysine residue in the active site of the dimeric form of the protein (Choi et al., 1987). Sheep brain PPOX is activated by tryptophan metabolites 3-hydroxyanthranilate and 3-hydroxykynurenine which bind to a regulatory domain distinct from the catalytic site. The structural domain with full catalytic activity composes approximately one-half of the molecular mass of PPOX, whereas the remaining portion contains the regulatory binding site (Kwon et al., 1991). Low concentrations of urea yield a rapidly formed “activated” species of the oxidase that is changed primarily at the active site in a manner that allows increased dissociation of substrate and product (Horiike et al., 1979).

Characteristics of PPOX in *Arabidopsis thaliana*

Only one PPOX protein has been reported in bacteria, yeast or animals (Salvo et al, 1998; Choi et al., 1983; Kazarinoff et al., 1975; Zhao and Winkler, 1995). However, wheat seedlings have at least three PPOX proteins, and two of these show PPOX activity (Tsuge et al., 1982). One isozyme was relatively more specific for PNP than PMP (9.22:1) and the other one was somewhat less specific (1.58:1) (Tsuge et al., 1982). One PPOX activity and the gene encoding this activity was identified in *Arabidopsis thaliana* (Sang et al., 2007). This PPOX can use both PNP and PMP as substrates, but with higher Km value for PNP than for PMP (Sang et al., 2007). The subunit of PPOX from bacteria, yeast and animals has 200-300 amino acids, which contains only a pyridox_oxidase domain. However PPOX from *Arabidopsis thaliana* is 530 amino acids long and has both a pyridox_oxidase domain and Yjef_N domain (Sang et al., 2007). Yjef_N domain exists widely in organisms. It is very well conserved, but its function is still not known.

The Yjef_N domain is fairly conserved across the three principal superkingdoms of life. It exists either as a single protein, or fused with other domains. A single copy of the Yjef_N domain is traceable to the last universal common ancestor of all life forms. Proteins with both Yjef_N domain and other domains can be found in archea, bacteria, fungi, plant, animals, and human.

In bacteria the Yjef_N domain is often found fused to a C-terminal kinase domain of the ribokinase superfamily, which suggests that the ancestral form of the Yjef_N domain may have functioned in the metabolism of a critical low molecular weight compound. Given that kinase domains are often fused to different phosphoesterase (phosphatase) domains, it is possible that the Yjef_N-type Rossman fold domains may also catalyze this reaction. The conservation of the acidic residues in the predicted active site of the Yjef_N domains is reminiscent of such residues in the active sites of diverse hydrolases. Thus, in the context of the decapping pathway, it is possible that the Yjef_N domains of Dcp3p and FLJ21128 catalyze hydrolytic RNA-processing reactions, such as, phosphoester hydrolysis, dephosphorylation, demethylation or glycosyl bond hydrolysis (Anantharaman and Aravind, 2004).

The screening for additional human YjeF_N domain containing proteins beside the apolipoprotein A-I interacting protein (AI-BP), identified two other genes designated hYjeF_N2-15q23 (formerly human homologue of yeast edc3) and hYjeF_N3-19p13.11 comprising the human YjeF_N family. Immunohistochemistry of human testes and ovaries showed an expression of hYjeF_N3-19p13.11 only in Leydig cells and theca cells, respectively, indicating a role in steroid hormone metabolism. Interestingly, the protein was also strongly expressed in Leydig cell tumors and in thecofibromas. The

identification of hYjeF_N2-15q23 in theca cells and granulosa cells in ovaries, in human spermatids of meiotic division part II and the apical membrane of Sertoli cells in testes suggest similar functions in oogenesis and sperm maturation which is strengthened by the identification of the spermatogenesis regulator HMGA1 as a conserved transcription factor. However, in contrast to AI-BP, both homologous proteins are unable to bind apoA-I. These results relate the human YjeF_N domain containing protein family to cholesterol processing and steroid hormone metabolism in spermiogenesis and oogenesis, and AI-BP may link this function to the HDL pathway (Rudolph et al., 2007).

Question mark for PPOX

All PPOX identified thus far need oxygen as the final electron acceptor. The question is how PNP oxidization to PLP occurs in anaerobically growing cells in which the PPOX presumably does not work. Two possibilities have been suggested (Salvo, et al., 2003). One possibility is that there is a single form of PdxH oxidase in wild-type *E. coli*, which uses oxygen aerobically and another electron acceptor anaerobically. A second possibility is that there is a second PPOX that functions under anaerobic conditions, but PdxH protein is required for expression or activation of this second anaerobic form of PPOX.

II IDENTIFICATION OF ATPPOX-1 FROM *ARABIDOPSIS THALIANA*

ABSTRACT

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) catalyzes the oxidative conversion of pyridoxamine 5'-phosphate (PMP) or pyridoxine 5'-phosphate (PNP) to pyridoxal 5'-phosphate (PLP). The *At5g49970* gene of *Arabidopsis thaliana* shows homology to PPOX's from a number of organisms including the *Saccharomyces cervisiae PDX3* gene. A cDNA corresponding to putative *A. thaliana* PPOX (*At*PPOX) was obtained using RT-PCR and primers landing at the start and stop codons of *At5g49970*. The putative *At*PPOX is 530 amino acid long and predicted to contain three distinct parts: a 64 amino acid long N-terminal putative chloroplast transit peptide, followed by a long Yjef_N domain of unknown function and a C-terminal Pyridox_oxidase domain. Recombinant proteins representing the C-terminal domain of *At*PPOX and *At*PPOX without transit peptide were expressed in *E. coli* and showed PPOX enzyme activity. The *PDX3* knockout yeast strain deficient in PPOX activity exhibited sensitivity to oxidative stress. Constructs of *AtPPOX* cDNA of different lengths complemented *PDX3* knockout yeast for oxidative stress. The role of the Yjef_N domain of *At*PPOX was not determined, but it shows homology with a number of conserved hypothetical proteins of unknown function.

INTRODUCTION

Vitamin B_6 is the collective term for a group of three related compounds, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated derivatives, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). All organisms require PLP and must either produce it or acquire it through their diet. PLP is an important cofactor in a wide range of biochemical reactions, including amino acid metabolism and antibiotic biosynthesis (Studart et al., 2005). Additionally, PLP or its derivatives may function as regulatory molecules in signal transduction regulating a number of membrane ion transporters (Hamasaki and Okubo, 1996; Palmieri et al., 2001; Salhany and Schopfer, 1993). Vitamin B_6 is also an efficient singlet oxygen quencher and potential antioxidant (Bilski et al, 2000). PLP is required for post-embryonic root development, and protects plant from high-salt, ultraviolet rays, osmotic and oxidative stresses (Shi et al, 2002; Chen and Xiong, 2005).

Most bacteria, fungi, and plants possess vitamin B_6 biosynthesis pathways, but mammals must be supplied with the vitamin in their diet (Tsuyoshi et al., 2004). Different vitamin B_6 biosynthetic pathways, referred to as the *de novo* pathway and the salvage pathway, are known. In *E. coli*, PNP is synthesized from the condensation of deoxyxylulose 5-phosphate and 4-hydroxythreonine-4-phosphate, catalyzed by PdxA and PdxJ (Laber et al., 1999), while plants, fungi and some other bacteria utilize ribose 5-phosphate or ribulose 5-phosphate and dihydroxyacetone phosphate or glyceraldehyde 3-phosphate to synthesize PLP (Studart et al., 2005; Raschle et al., 2005).

The vitamin B_6 salvage pathway is involved in interconvertions between different vitameric forms of vitamin B_6. In vitamin B_6 auxotrophic organisms, uptake of PN, PL, or PM from the extracellular space is required to generate intracellular PLP via the salvage pathway. Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX), pyridoxal kinase, pyridoxal reductase, and vitamin B_6 phosphatase are all involved in the salvage pathway of plants (Tsuyoshi et al., 2004). However, only pyridoxal kinase in this pathway has been studied in detail (Shi and Xiong, 2002; Lum et al., 2002). This enzyme catalyzes the transfer of a phosphate from ATP to vitamin B_6 vitamers resulting in corresponding phosphorylated derivatives (Kerry et al., 1986). PPOX plays an important role in the salvage pathway because it converts PMP or PNP to PLP, the active form of vitamin B_6 (Salvo et al., 1998). PPOX has been identified in *S. cerevisiae*, human, mammalian cells, *E. coli* and several other bacteria (Lam and Winkler, 1992; Loubbardi et al., 1995; Kang et al., 2004; Bahn et al., 2002), but little information is available about PPOX in plants. Two isozymes of PPOX from wheat seedlings were partially purified. One isozyme used only pyridoxine 5'-phosphate as substrate, while the other one used both PNP and PMP with approximately equal efficiency (Tsuge et al., 1982).

Here we present isolation and characterization of an *A. thaliana* cDNA encoding a putative pyridoxine (pyridoxamine) 5'-phosphate oxidase, and the functional characterization of various parts of a recombinant protein made from this cDNA. The plant protein also is functionally sufficient to complement the abatement of oxidative stress in yeast cells lacking a functional PPOX gene.

RESULTS AND DISCUSSION

Isolation of *A. thaliana* pyridoxine (pyridoxamine) 5'- phosphate oxidase cDNA and its homology to S. cerevisiae PDX3

PDX3 showed PPOX activity in *S. cerevisiae* (Loubbardi et al., 1995). BLASTP analysis using PDX3 demonstrated a putative PPOX protein in *A. thaliana*. PDX3 shows homology with the C-terminal half of *At*PPOX encoded by *At5g49970*. The C-terminal half of *At*PPOX shares 44% amino acid sequence identity and 61% similarity with *S. cerevisiae* PDX3 (Fig. 1A). Additionally, the *At*PPOX amino acid sequence shows roughly the same level of homology with a number of PPOX genes from a wide range of bacteria, fungi, and lower and higher animals.

The cDNA corresponding to the *AT5G49970* gene was amplified using RT-PCR with primers that include the putative start and stop codons. The 1593 bp cDNA sequence is in full length and encodes a 530 amino acid polypeptide that contains three significant features. The N-terminal 64 amino acids are identifiable as a putative chloroplast transit peptide (http://www.cbs.dtu.dk/services/ChloroP/). A Yjef_N domain of unknown function can be identified at amino acid position 100 to 281. And the C-terminal region contains a Pyridox_oxidase domain starting at amino acid position 364 and ending at position 454 (Fig. 1B).

Amino acid sequence of *At*PPOX has unique Yjef_N domain. Both the rice and *Giardia lamblia* PPOXes have amino acid sequence similar to *At*PPOX that contain a Yjef_N domain (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The human Yjef_N domain shows homology to apolipoprotein A-I binding protein and may be involved in the

regulation of vesicle fusion in the endosomal/lysosomal route (Schmitz and Rudolph, 2004). The Yjef_N domain also shows homology to human TGR-CL10C thyroidal receptor for N-acetylglucosamine (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). YNL200C in *S. cerevisiae* carrying Yjef_N domain homologue is a hypothetical protein and shows similarity to *E. coli* hydroxyethylthiazole kinase at the C-terminus and to enzymes involved in thiamine biosynthesis, and it may be involved in interactions with other Pfam family proteins (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF03853). The function of Yjef_N domain in *At*PPOX remains to be elucidated.

Expression of the recombinant AtPPOX in E. coli and assay of PPOX activity

To determine the PNP/PMP oxidase activity of putative *At*PPOX, constructs of different length were expressed in *E. coli*. The recombinant proteins were derived from the full coding sequence (*AtPPOX-1593*), the *AtPPOX* without the putative chloroplast transit peptide sequence (*AtPPOX-1401*), and the C-terminal pyridox_oxidase domain sequence (*AtPPOX-681*). *AtPPOX-1401* and *AtPPOX-681* were highly expressed (Fig. 2), but recombinant protein from the full length *AtPPOX-1593* was not detected.

The expressed recombinant proteins were purified using the Probond™ purification system, and both the total protein extracts and purified recombinant proteins were analyzed utilizing SDS-PAGE (Fig. 2). Recombinant protein from *AtPPOX-1401* and from *AtPPOX-681* tagged with 6×His and Xpress™ epitope had a theoretical molecular weight of 56 kDa and 30 kDa, respectively. The molecular determined from the SDS-PAGE were consistent with this inferred theoretically.

The purified recombinant proteins encoded by *AtPPOX-1401* and *AtPPOX-681* used both PNP and PMP as substrates, but the relative rate of catalytic activity for PNP was about 3.4 times higher than for PMP (Table 1). When the molecular size of each protein is taken into account, the specific activity of the *AtPPOX-681* derived protein is approximately 17% higher than that of the *AtPPOX-1401* derived protein. These results suggest that the C-terminal pyridox_oxidase domain has PPOX activity with PNP and PMP, and that the activity is independent of the N-terminal Yjef_N domain regardless of the substrate utilized.

Complementation of YBR035C for oxidative stress tolerance by AtPPOX

Since pyridoxine vitamers act as singlet oxygen scavengers [5], the PDX3 deletion strain (YBR035C) was grown in different concentrations of hydrogen peroxide. In YPD liquid medium containing 2mM H_2O_2, YBR035C showed a distinct growth defect compared to the wild type BY4743 strain (Fig. 3). Yeast shuttle plasmids, p426GPD/*AtPPOX-1593*, p426GPD /*AtPPOX-1401* and p426GPD / *AtPPOX-681* were introduced into YBR035C strain of yeast. As a control, the p426GPD empty vector was also introduced. In YPD liquid media without H_2O_2, no differences in the growth of control strain (BY4743), PDX3 deletion strain (YBR035C) and four different transformants were demonstrated. While in YPD medium with 2mM H_2O_2, YBR035C and those transformed with p426GPD empty vector did not grow, whereas YBR035C transformed with p426GPD/*AtPPOX-1593*, p426GPD /*AtPPOX-1401* and p426GPD /*AtPPOX-681* constructs showed significant growth (Fig. 3). Complementation efficiency was calculated from the growth of control strain BY4743 under identical growth

conditions. The efficiency of complementation was 89% for *AtPPOX-681* followed by *AtPPOX-1401* and *AtPPOX-1593* showing 86% and 60% complementation, respectively. The complementation result reaffirmed measurement of PPOX enzyme activity of the recombinant proteins and provided support for the independent function of C-terminal pyridox_oxidase domain of *At*PPOX in the catalytic activity.

MATERIAL AND METHODS

RNA extraction and cDNA cloning

Two-week-old *A. thaliana* seedlings were ground to a powder in liquid nitrogen. RNA was extracted using RNAeasy plant mini kit (Qiagen), as described in the instruction manual. Total RNA (0.5µg) was reverse-transcribed using the one-step RT-PCR kit (Qiagen) in a 50 µl RT-PCR reaction mixture. PCR primers used were 5'-ATGAGGAATGTGATACGCAGAGTC-3' and 5'-TCATGGGGCCAATCTATGAA-3'. Amplification was performed in a thermal-cycler as follows: 30 min at 50°C; 15 min at 95°C; then 1 min at 94°C; 1 min at 55°C; 1 and a half min at 72°C for 30 cycles, followed by 10 min at 72°C. The single RT-PCR product of 1593 bp was observed in agarose gel electrophoresis and cloned into the PGEM-T Easy vector (Promega). The sequence of both strands of the amplified DNA was verified.

Expression of recombinant protein and purification of the AtPPOX-1

A set of primers, 5'-ATGCAAGATTCAGGATCACCAC-3' and 5'-TCATGGGGCCAACTATGAA-3' was used to amplify the AtPPOX-1 without putative

transit peptide sequence, and the resulting PCR product was 1401 bp long. Another pair of primers, 5'-ATGCAAGATTCAGGATCACCAC-3' and 5'-GTCGACTTAAATTC TAACACAC-3' was used to amplify the C-terminal pyridox-domain of the AtPPOX-1 resulting in a PCR product of 681 bp. The 1593 bp, 1401 bp, and 681 bp fragments were TA cloned into the pTrcHis-TOPO expression vector (Invitrogen) for expression of the recombinant proteins fused to the C-terminus of XpressTM epitope and hexa-histidine tag. These three plasmids were designated as pTrcHis-TOPO/AtPPOX-1593, pTrcHis-TOPO/AtPPOX-1401 and pTrcHis-TOPO/ AtPPOX-681, respectively. The expression of recombinant proteins from these constructs were induced by the addition of 1 mM isopropyl-1-β-thio-D-galactopyranoside (IPTG) when the culture reached an OD_{600} = 0.5, followed by growth for 5h at 37°C. The recombinant proteins were purified using the ProBond™ purification System (Invitrogen) under nondenaturing conditions as described by the manufacturer.

Assay of AtPPOX enzyme activity

PMP was obtained from Sigma Chemical Co. PNP was synthesized by reducing PLP with sodium borohydride (Musayev et al., 2003). PNP and PMP oxidase activities were measured by monitoring PLP formation in Tris-phosphate buffer as described by Zhao *et al* (Zhao and Winkler, 1995). At 414 nm, the Schiff base formed between Tris and PLP has an extinction coefficient of 5900 $M^{-1}cm^{-1}$. Reaction mixtures (1.0 ml) contained 0.2 M Tris-HCl, 0.2 M KPO_4 (pH 8.5), 0.1 mg BSA, 2.0 µM FMN, 0.5 mM PMP or PNP, and 20 to 30 µg purified recombinant proteins. The enriched 6×His Xpress™ epitope was used as control for the enzyme assay, and the assay blanks

contained the same as reaction mixture except that no enzyme was added. Initial velocities were measured by monitoring the constant increase in A_{414} for 5min at 37°C. No more than 3% of the substrate was used during this incubation. One unit of specific activity was defined as the formation of 1μmol of PLP per min at 37°C. Protein concentrations were determined using Bradford microassay method with BSA (1 μg/ml-20 μg/ml) as the standard (Bradford MM, 1976).

Yeast complementation

The *S. cerevisiae* wild type control strain BY4743 and *PDX3* deletion strain YBR035C were obtained from American Type Culture Collection (Manassas, VA, USA). Amplified PCR products of 1593 bp, 1401 bp and 681 bp fragments cloned in PGEM-T Easy vector (Promega) were isolated by *Eco*R1 digestion, and subcloned into the *Eco*R1 site of p426GPD yeast shuttle vector. Three resulting plasmids were designated as p426GPD/*AtPPOX-1593*, p426GPD /*AtPPOX-1401* and p426GPD /*AtPPOX-681*, respectively. These three plasmids were transferred into YBR035C respectively by the heat shock method (Gietz and Woods, 1994). Complementation for sensitivity to oxidative stress was carried out in YPD liquid medium with 2mM H_2O_2. The complementation efficiency was calculated by dividing the OD_{600nm} of the complemented yeast strain by that of the control strain BY4743 during logarithmic phase.

Table 1. PNP/PMP oxidase activities of the purified recombinant proteins, *At*PPOX-1401 and *At*PPOX-681, for PMP and PNP*

Purified Recombinant Protein	Specific Enzyme Activity				Activity Ratio (PNP/PMP)
	PNP		PMP		
	units/mg	units/pmole	units/mg	units/pmole	
***At*PPOX-1401**	**154.6**	**8.7**	**45.8**	**2.6**	**3.4**
***At*PPOX-681**	**338.1**	**10.1**	**101.2**	**3.1**	**3.3**

*** Details about enzyme expression and purification, enzyme assay, and unit definition are in Materials and Methods.**

Figure 1. (A) Amino acid sequence alignment of *At*PPOX and yeast PDX3. Amino acids identical in these two proteins are highlighted in black, and conservative substitutions are highlighted in gray. Putative transit peptide is shown in bold and the Yjef_N domain is underlined. The CLUSTALW program (Thompson et al., 1994) was used for sequence alignment. (B) Schematic diagram of predicted domains of *At*PPOX. *At*PPOX contains a putative chloroplast transit peptide, N-terminal YjeF_N domain and C-terminal Pyridox_oxidase domain.

A

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AtPPOX        1   MRNVIRRVTTMTFTFLLQSPPLPISPSPPQFSLSSSPLSKTQRFITPSQGSRLRTLCTKV
yeastPDX3     1   ------------------------------------------------------------

AtPPOX       61   IIPNMQDSGSPPLSYLTQREAAEIDETLMGPLGFSIDQLMELAGLSVAASIAEVYKPEEY
yeastPDX3     1   ------------------------------------------------------------

AtPPOX      121   SRVLAICGPGNNGGDGLVAARHLHHFGYKPFICYPKRTAKPLYTGLVTQLDSLSVPFVSV
yeastPDX3     1   ------------------------------------------------------------

AtPPOX      181   EDLPDDLSKDFDVIVDAMFGFSFHGAPRPPFDDLIRRLVSLQNYEQTLQKHPVIVSVDIP
yeastPDX3     1   ------------------------------------------------------------

AtPPOX      241   SGWHVEEGDHEDGGIKPDMLVSLTAPKLCAKRFRGPHHFLGGRFVPPSVAEKYKLELPSY
yeastPDX3     1   ------------------------------------------------------------

AtPPOX      301   PGTSMCVRIGKPPKVDISAMRVNYVSPELLEEQVETDPTVQFRKWFDEAVAAGLRETN--
yeastPDX3     1   -MTKQAEETQKP--IIFAPETYQYDKFTLNEKQLTDDPIDLFTKWFNEAKED-PRETLPE

AtPPOX      359   AMALSTANKDK-KPSSRMVLLKGFDENGFVWFTNY-ESKKGSDLSENPSAALLFYWEILN
yeastPDX3    57   AITFSSAELPSGRVSSRILLFKELDHRGFTIYSNWGTSRKAHDIATNPNAAIVFFWKDLQ

AtPPOX      417   RQVRIEGPVERIPESESENYFHSRPRGSQIGAIVSKQSSVVPGRHVLYDEYEELTKQYSD
yeastPDX3   117   RQVRVEGITEHVNRETSERYFKTRPRGSKIGAWASRQSDVIKNREELDELTQKNTERFKD

AtPPOX      477         GSVIPKPKNWGGFRLKPNLFEFWQGQPSRLHDRLQYSLQDVNGNPAWKIHRLAP
yeastPDX3   177         AEDIPCPDYWGGLRIVPLEIEFWQGRPSRLHDRFVYRRKTEND--PWKVVRLAP
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B

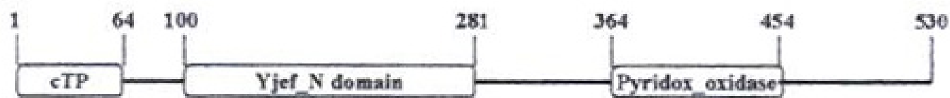


Figure 2. SDS-PAGE of recombinant *At*PPOX protein of different lengths separated on a 12.5% gel. Lanes: 1, crude cell lysate of *E. coli* transformed with pTrcHis-TOPO/*AtPPOX-1401*; 2, purified *At*PPOX-1401 recombinant protein; 3, crude cell lysate of *E. coli* transformed with pTrcHis-TOPO/*AtPPOX-681*; 4, purified *At*PPOX-681 recombinant protein; 5, crude extract of *E. coli* transformed with pTrcHis-TOPO vector.

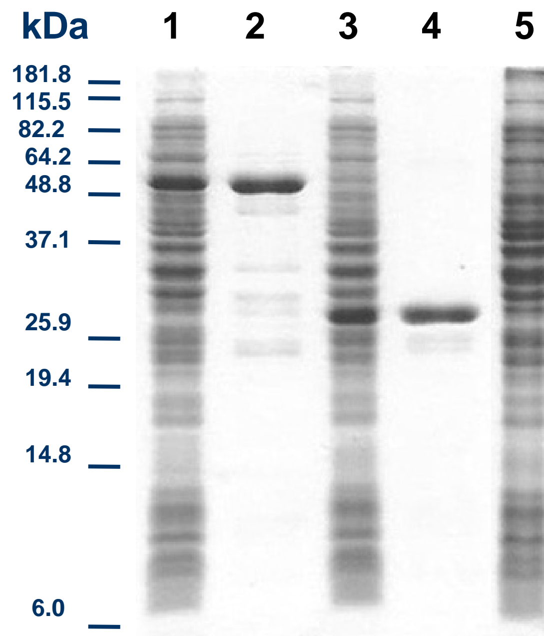
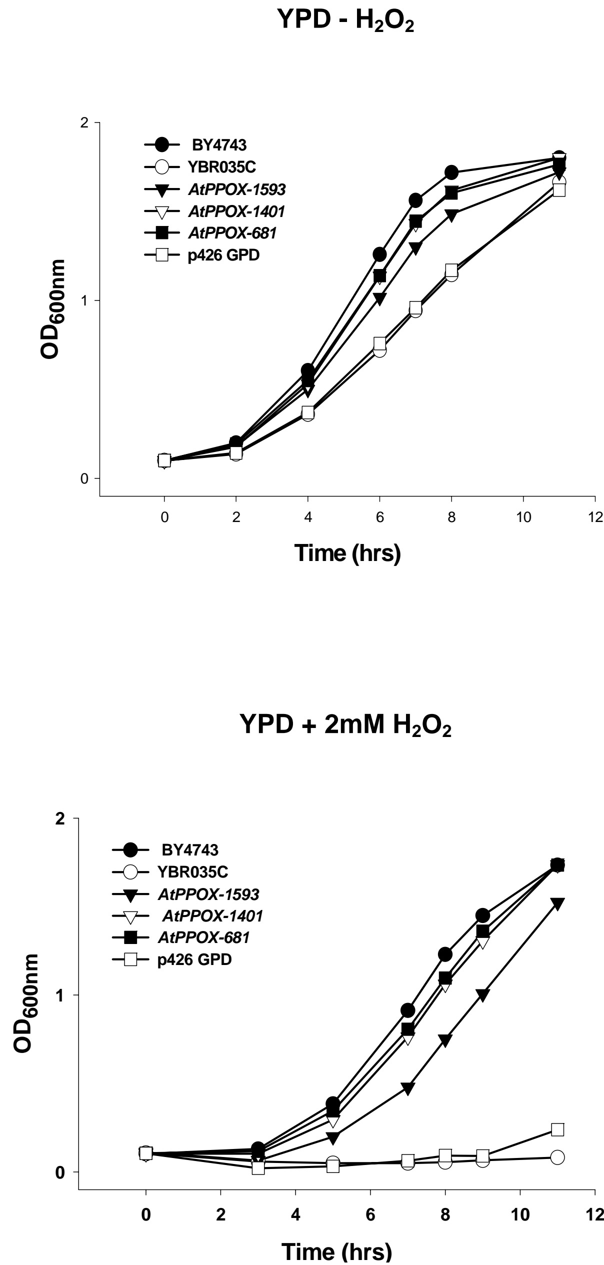


Figure 3. Complementation of YBR035C under oxidative stress with *At*PPOX constructs. BY4743, YBR035C and YBR035C transformed with vector p426GPD, p426GPD /*AtPPOX-1593*, p426GPD /*AtPPOX-1401* and p426GPD /*AtPPOX-681*, respectively were grown in liquid YPD medium without H_2O_2 (top) or with 2mM H_2O_2 (bottom).



III IDENTIFICATION OF ATPPOX-2 FROM *ARABIDOPSIS THALIANA*

ABSTRACT

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) is an important enzyme involved in the salvage pathway of vitamin B_6 biosynthesis. This enzyme converts pyridoxine 5'-phosphate (PNP) or pyridoxamine 5'-phosphate (PMP) into pyridoxal 5'-phosphate (PLP), a cofactor for numerous enzymes. Using RT-PCR, we cloned the cDNA of *AtPPOX*-2, a second putative PPOX gene at the locus of *At2g46580* in *Arabidopsis thaliana*. The RT-PCR amplified cDNA was cloned into an *E. coli* expression vector and a yeast shuttle vector. Both PPOX enzyme assay and the complementation of PDX3 knockout yeast for oxidative stress showed that the gene at locus *At2g46580* encodes for a PPOX (AtPPOX-2) with low PPOX specific activity. The catalytic efficiency of AtPPOX-1 is almost 300-fold higher than that of AtPPOX-2. Based on bioinformatic analysis, there is a putative transit peptide at the N-terminus. The truncated AtPPOX-2 without 18 amino acids at the N-terminal end lost PPOX activity suggesting that the N-terminal amino acids are necessary for the enzyme activity of AtPPOX-2.

INTRODUCTION

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) catalyzes the oxidation of pyridoxine 5'-phosphate (PNP) and pyridoxamine 5'-phosphate (PMP) into pyridoxal 5'-phosphate (PLP) (Wada and Snell, 1961; Yang and Schirch, 2000). PLP is the active form of vitamin B6, acting as a ubiquitous, essential cofactor for numerous enzymes involved in amino acid metabolism and several other metabolic pathways. Plants and fungi can synthesize PLP directly via PLP synthase complex (Studart et al., 2005). In mammalian cells where vitamin B_6 is a nutritional requirement and acquired from their nutrients, PPOX is especially important for the biosynthesis of PLP through the PLP salvage pathway.

PPOX exists in almost all organisms. Wheat seedlings have at least two PPOX enzymes (Tsuge et. al., 1982) while bacteria, fungi and animals appear to have only one PPOX enzyme (Zhao and Winkler, 1995; Kazarinoff et. al., 1975; Choi and Churchich, 1987). Two isozymes of PPOX from wheat seedlings were partially purified. One isozyme used only pyridoxine 5'-phosphate as substrate, while the other one used both PNP and PMP with approximately equal efficiency. PNP is by far the better substrate for the PPOX from *E. coli*, whereas PPOX from rabbit liver oxidizes both substrates to almost the same extent (Zhao and Winkler, 1995; Choi et al., 1983). AtPPOX-1 encoded by *At5g49970* in *Arabidopsis thaliana* oxidizes both PNP and PMP, with higher efficiency towards PNP (Sang et al., 2007).

Bioinformatic analysis of *A. thaliana* genome suggested the presence of an additional gene at the *At2g46580* locus which also contains a pyridox_oxidase superfamily domain, but there is no biochemical evidence to support its function as PPOX enzyme. In this

study, we report that *At2g46580* encodes for a second PPOX in *A. thaliana* (AtPPOX-2). The catalytic efficiency of AtPPOX-2 is about 300-fold lower than that of AtPPOX-1.

RESULTS

Cloning of cDNA at the *At2g46580* locus in *A. thaliana*

Bioinformatically, the protein encoded by *At2g46580* locus in *A. thaliana* showed homology to the Pyridox_oxidase domain found in pyridoxine 5'-phosphate oxidase-related proteins in the NCBI data base. In order to determine the function of protein encoded by the *At2g46580* locus, the corresponding cDNA was amplified by a one-step RT-PCR using the total RNA as template. The primers included the first 22 nucleotides of the ORF, including the ATG start codon, and the last 23 nucleotides, including the stop codon. Amplified PCR products were cloned into the PGEM-T Easy vector and both strands were sequenced. The full length cDNA is 597 base pairs long and encodes for a 198 amino acid polypeptide.

Expression and purification of the recombinant protein AtPPOX-2

To express AtPPOX-2, its cDNA sequence without a stop codon was first cloned into the PGEM-T Easy vector, and then subcloned into the pET-20b expression vector. The resulting pET-20b/*AtPPOX-2* plasmid was transformed into C41 pLys host cells. When induced by IPTG at room temperature, the recombinant protein was highly expressed. Since the recombinant protein has a C-terminal His tag, it was purified using the Probond^{TM} purification system. The purified recombinant proteins and the total protein extracts were analyzed utilizing SDS-PAGE (Fig. 1). Recombinant AtPPOX-2

protein tagged with 6×His had a molecular weight of 28 kDa. The molecular weight inferred from the SDS-PAGE was consistent with that obtained theoretically.

PPOX activity of the recombinant AtPPOX-2

To determine if the purified protein has PPOX activity, the Schiff base method described by Zhao et al. (1995) was used. But PPOX activity was not detected in the recombinant protein. However, we were able to measure the PPOX activity using a method described by Tsuge et al. (1982). This result demonstrated that the recombinant putative AtPPOX-2 protein had low PPOX activity. Controls for each enzyme assay did not show any activity. We previously had found and characterized a PPOX in *Arabidopsis thaliana* (Sang et al., 2007) encoded by the *A5g49970* gene which we named as AtPPOX-1. Similar to AtPPOX-1, AtPPOX-2 can accept both PNP and PMP as substrate and the catalytic activity for PNP was higher than for PMP. However, the catalytic efficiency of AtPPOX-1 is almost 300-fold higher than that of AtPPOX-2. PPOX activity of AtPPOX-1 and AtPPOX-2 measured using the method developed by Tsuge et al. is shown in Figure 2. The truncated AtPPOX-2 without the N-terminal 18 amino acids was also expressed and purified, but it has no enzyme activity (data not shown). This result demonstrated that the N-terminal 18 amino acids are necessary for PPOX activity of AtPPOX-2.

Complementation of yeast YBR035C for oxidative stress tolerance by AtPPOX-2

PDX3 is a yeast gene coding for yeast PPOX protein. The yeast *PDX*3 deletion strain, YBR035C, showed a distinct growth defect compared to the wild type BY4743

strain when grown in YPD liquid medium containing 2mM H_2O_2 (Sang et al., 2007). To confirm if *AtPPOX*-2 encoded protein has PPOX activity, full length cDNA was cloned into the p426GPD yeast shuttle vector, and the resulting construct p426GPD/AtPPOX-2 was used to transform YBR035C yeast strain. As a control, the p426GPD empty vector was transformed into the YBR035C yeast strain. On SCM media without 2mM H_2O_2, BY4743, YBR035C and the transformants with p426GPD/AtPPOX-2 plasmid or empty p426GPD grew almost the same. On SCM media supplemented with 2mM H_2O_2, YBR035C control and the YBR035C transformed with empty p426GPD vector did not show appreciable growth, whereas the YBR035C transformed with p426GPD/AtPPOX-2 construct showed some growth (Figure 3). This result demonstrated a weak complementation of PDX3 by AtPPOX-2. This result is consistent with the enzyme assay.

DISCUSSION

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) has been widely found in bacteria, fungi, animals and plants. Except for wheat seedlings, no other organisms have been reported to have more than one PPOX isozyme. Wheat seedlings were reported to have three PPOX isozymes (Tsuge et al., 1982). We reported the first PPOX encoded by *At5g49970* in *A. thaliana* (Sang et al., 2007). Herein, a second PPOX isozyme encoded by *At2g46580* in *A. thaliana* is identified.

PPOX from different organisms catalyze PMP and PNP oxidation at different catalytic efficiency. PNP is by far the better substrate for the PPOX from *E. coli* (Zhao and Winkler, 1995; Salvo et. al. 1998). PPOX from rabbit liver oxidizes both substrates to almost the same extent (Choi et al., 1983). Wheat seedlings were reported to have at

least three PPOX isozymes, two of which were partially purified. One of the PPOX isozymes oxidized both PNP and PMP at approximately equal rates, whereas the other used only PNP as substrate (Tsuge et al., 1982). However, in *Arabidopsis thaliana*, both AtPPOX-1 and AtPPOX-2 can accept PNP and PMP as substrate, and have higher catalytic efficiency toward PNP.

All PPOX studied thus far need oxygen as the final electron acceptor. The question is how PNP oxidization to PLP occurs in anaerobically growing cells in which the PPOX presumably does not work. Two possibilities have been suggested (Salvo et al., 2003). One possibility is that there is a single form of PdxH oxidase in wild-type *E. coli*, which uses oxygen aerobically and another electron acceptor anaerobically. A second possibility is that there is a second PPOX that functions anaerobically. Compared to AtPPOX-1, AtPPOX-2 has a much lower PPOX activity in *vitro.* It is possible that AtPPOX-2 needs the specific final electron acceptor or other factors that do not exist in *vitro* conditions. It is also likely that the recombinant AtPPOX-2 expressed in *E. coli* does not have the correct modifications which may occur in plants.

MATERIAL AND METHODS

cDNA cloning of *AtPPOX*-2

RNAeasy plant mini kit (Qiagen) was used to extract the total RNA from the two-week-old *A. thaliana* seedlings, as described in the instruction manual. Total RNA (0.5µg) was reverse-transcribed using the one-step RT-PCR kit (Qiagen) in a 50 µl RT-PCR reaction mixture. PCR primers used were 5'-ATGGGAACACATGTAGCACCAT-3' and 5'-TTATGGGTTAACCTTTTCTGAAGTC-3'. Amplification was performed in a

thermal-cycler as follows: 30 min at 50°C; 15 min at 95°C; then 1 min at 94°C; 1 min at 52°C; 1 min at 72°C for 30 cycles, followed by 10 min at 72°C. The single RT-PCR product of 597 bp was observed in agarose gel electrophoresis. This fragment was cloned into the PGEM-T Easy vector (Promega). The sequence of the amplified DNA was verified.

Expression and purification of the recombinant AtPPOX-2

A set of primers, 5'-GGATCCATGGGAACACATGTAGCACCAT-3' and 5'-GAATCCTGGGTTAACCTTTTCTGAAGTC-3' was used to amplify the cDNA of *At2g46580*. The 606 bp fragments were TA cloned into the PGEM-T Easy vector. The fragments were cut out with *Bam*H1 and *Eco*R1 and cloned into the pET-20b expression vector (Novagen) for expression of the recombinant proteins fused to the C-terminus hexa-histidine tag. The resulting plasmid was designated as pET-20b/AtPPOX-2 and was used to transform *E. coli* strain, C41 pLys. The transformed *E. coli* culture was induced by the addition of 1 mM isopropyl-1-β-thio-D-galactopyranoside (IPTG) when the culture reached an OD_{600} = 0.5, followed by growth for 5 hrs at 26°C. Cell lysate was used to purify the recombinant proteins using the ProBond^{TM} purification System (Invitrogen) under nondenaturing conditions as described by the manufacturer.

Enzyme Assay of the recombinant AtPPOX-2

The activity of PPOX was measured with a colorimetric procedure (González et al., 2007). Enzyme activity of purified recombinant protein was determined in 0.5-mL reaction mixtures containing 200 mM Tris-HCl, 200 mM KPO_4, pH 8.5, and 0.2 mM

PMP/PNP. After 1 h of incubation at 37°C, reactions were stopped by addition of 50 μL of chilled 50% (w/v) TCA. The reaction mixture was centrifuged at 4°C and 1,500 rcf for 10 min, and supernatant was transferred to a clean tube. PLP formation was measured at OD_{410} following addition of 2% (w/v) phenylhydrazine in 10 N H_2SO_4 and incubation on ice for 30 min. Protein concentration was determined with the Bradford protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as the standard.

Complementation of PDX3 deletion strain for oxidative tress

The *S. cerevisiae* wild type control strain BY4743 and PDX3 deletion strain YBR035C were obtained from American Type Culture Collection (Manassas, VA, USA). Amplified PCR products of 597 bp fragments cloned in PGEM-T Easy vector (Promega) were isolated by *Eco*R1 digestion, and subcloned into the *Eco*R1 site of p426GPD yeast shuttle vector. The resulting p426GPD/AtPPOX-2 plasmid was transferred into YBR035C by the heat shock method (Gietz et al., 1994). Complementation for sensitivity to oxidative stress was carried out on SCM medium plate supplemented with 2mM H_2O_2 as described by Sang et al. (2007).

Figure 1. SDS-PAGE of the recombinant AtPPOX-2 separated on a 12.5% SDS-Polyacrylamide gel. Lanes: 1, purified recombinant AtPPOX-2; 2, crude extract of C41 pLys host cells transformed with Empty pET-20b vector; 3, crude cell lysate of C41 pLys host cells transformed with pET-20b/AtPPOX-2.

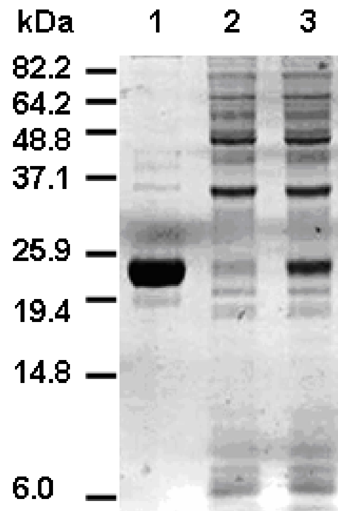
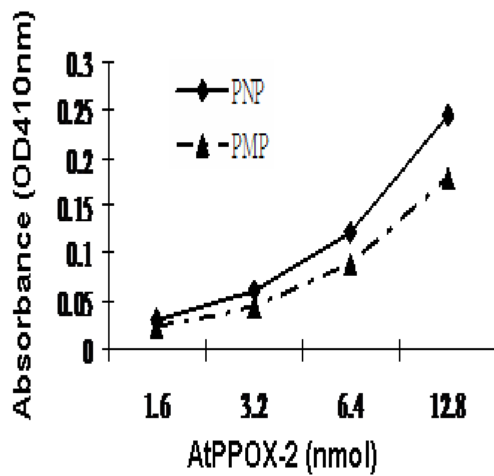


Figure 2. PPOX activity of the purified recombinant AtPPOX-1 and AtPPOX-2 with PMP and PNP as substrates. The enzyme activity was measured by determining the amount of PLP formed with a colorimetric assay after 1 hour incubation of enzyme reactions containing increasing amounts of total protein.



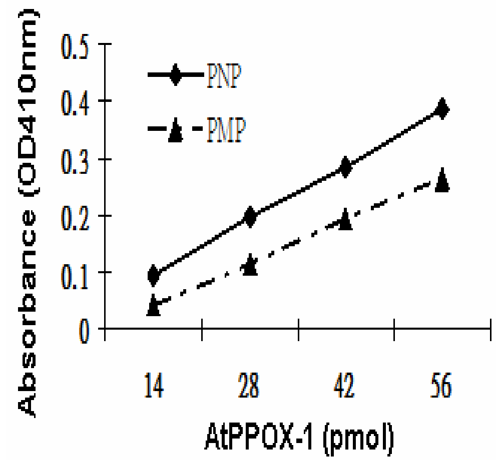
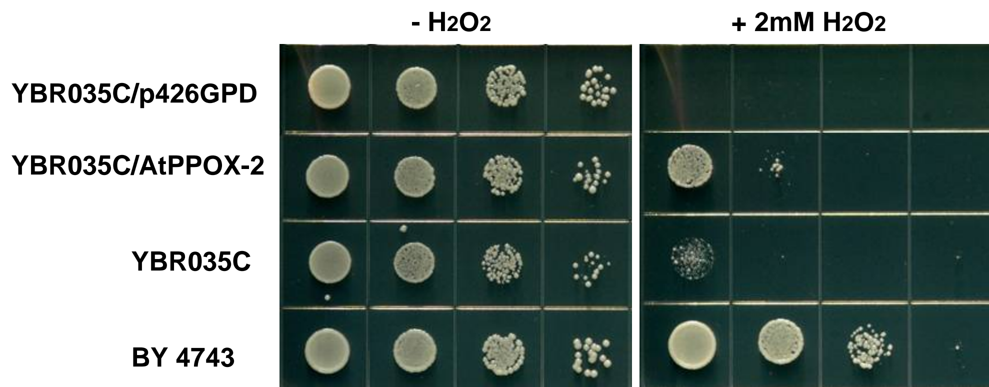


Figure 3. Complementation of the YBR035C under oxidative stress by AtPPOX-2. BY4743, YBR035C, YBR035C transformed with p426GPD/AtPPOX-2, and YBR035C transformed with empty vector p426GPD control were grown on SCM - H_2O_2 (left) or +2mM H_2O_2 (right). Each row represents a serial 10-fold dilution from a starter culture with an OD of 0.01at 600 nm. Stationary phase cultures (12 hrs) were diluted to an optical density (OD_{600}) of 0.01.



IV EVOLUTIONARY ANALYSIS OF ATPPOX-1 AND ATPPOX-2 FROM *ARABIDOPSIS THALIANA*

ABSTRACT

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) is an important enzyme involved in the salvage pathway of vitamin B6 biosynthesis. It converts pyridoxine 5'-phosphate (PNP) or pyridoxamine 5'-phosphate (PMP) into pyridoxal 5'-phosphate (PLP), a cofactor for numerous enzymes. Two PPOX proteins have been identified in *Arabidopsis thaliana*, which show considerable difference in size of the mature protein and amino acid sequence. Phylogenetic analysis of AtPPOX-1 and AtPPOX-2 homologs across all domains of life suggests that AtPPOX-1 and AtPPOX-2 have independent origins. AtPPOX-1 phylogeny appears congruent with the underlying branching pattern of the overall tree of life, while AtPPOX-2 phylogeny suggest that plant PPOX-2 may have originated from cyanobacteria. AtPPOX-1 homologs from over 30 plant species have an additional Yjef_N domain preceding Pyridox_Oxidase domain at the C-terminal end. AtPPOX-1 homologs from bacteria, fungi and animals have only the Pyridox_Oxidase domain. Presence of Yjef_N domain in land plants in AtPPOX-1 homologs suggest that acquisition of this domain and its fusion with pyridox_oxidase domain began with the endosymbiotic acquisition of the chloroplast.

INTRODUCTION

Pyridoxine (Pyridoxamine) 5'-phosphate oxidase (PPOX) converts pyridoxine 5'-phosphate (PNP) or pyridoxamine 5'-phosphate (PMP) into pyridoxal 5'-phosphate (PLP), a cofactor for numerous enzymes, and is found in all organisms. Both the prokaryotic and eukaryotic enzymes are homodimers with one molecule of FMN tightly binding to each subunit (Kazarinoff et al., 1975; Choi et al., 1983; Zhao et al., 1995; Salvo et al., 1998). It was thought that the subunit size of both the prokaryotic and eukaryotic PPOX is 25-28 kDa (Salvo et al., 1998). However, AtPPOX-1 from *A. thaliana* is 530 amino acids long and has a molecular weight of 62 kDa (Sang et al., 2007). Besides the pyridox_oxidase domain present in all PPOX, AtPPOX-1 has an extra Yjef_N domain. Truncated recombinant AtPPOX-1 without a Yjef_N domain has full enzyme activity, suggesting that PPOX activity of AtPPOX-1 is independent of the Yjef_N domain. AtPPOX-2 is a considerably smaller protein with 24 kDa size. AtPPOX-2 has pyridox_oxidase domain and does not have Yjef_N domain at its N-terminal.

In order to determine the evolutionary relationship of two very dissimilar proteins, i.e. AtPPOX-1 and AtPPOX-2 in *A. thaliana*, and to determine the stage when the yjef_N domain began to fuse with pyridox_oxidase domain in AtPPOX-1 homologs, various genomic databases were searched for AtPPOX-1 and AtPPOX-2 homolog from various organisms. Using the retrieved protein sequences, phylogenetic analysis was carried out to analyze the evolutionary history of AtPPOX-1 and AtPPOX-2.

RESULTS

AtPPOX-1 and AtPPOX-2 have low similarity

Since AtPPOX-1 and AtPPOX-2 both have PPOX activity, amino acid sequence alignment was performed to determine similarities between these two proteins (Figure 1). The amino acid sequences of these two PPOX show 37% similarities only in the ppox_domain of the protein.

AtPPOX-1 and AtPPOX-2 homologs retrieved from various databases

All available homologs of AtPPOX-1 and AtPPOX-2 were retrieved from protein, EST, and genome databases across all domains of life. Most sequences used here are available from the NCBI protein database, but some were reconstructed from EST and genomic sequence. Complete coding sequences were retrieved for most proteins, while for some only partial sequences were obtained. Overall, 144 AtPPOX-1 homologs (Table 1) and 56 AtPPOX-2 homologs (Table 2) were retrieved. Some organisms have both AtPPOX-1 and AtPPOX-2 homologs, but only one of them was found in many organisms. Animals have only the AtPPOX-1 homolog. Prasinophyte algae, such as *Ostreococcus tauri* and *Ostreococcus lucimarinus* possess only AtPPOX-2 homolog. However, no AtPPOX-1 or AtPPOX-2 homolog was found in Archea.

Conserved regions among all AtPPOX-1 and AtPPOX-2 homologs

To determine the conserved regions among all PPOX proteins, one amino acid sequence of AtPPOX-1 and AtPPOX-2 homologs from each domain of life was aligned. It was found that there are three conserved regions among all PPOX (Figure 2). In terms

of AtPPOX-2, R_{42}, V_{44}, V_{45}, P_{88}, P_{90}, P_{93}, P_{191} and P_{198} are conserved among all PPOX proteins.

AtPPOX-1 homologs from plants have both yjef_N and pyridox_oxidase domain

Similar to AtPPOX-1, AtPPOX-1 homologs from *Vitis vinifera*, *Oryza sativa*, *Picea sitchensis* and P*hyscomitrella patens* found in NCBI protein database are also much longer than PPOX found in bacteria, fungi, and animals. These proteins from plants not only have pyridox_oxidase domain but also have an extra Yjef_N domain, whereas AtPPOX-1 homologs from other domains of life have only pyridox_oxidase domain. In order to determine if all plant AtPPOX-1 homologs have both domains, AtPPOX-1 homologs retrieved from database searches were analyzed. Interestingly, AtPPOX-1 homologs from all land plants, Chlorophyte algae, Charaphyte algae and red algae have both yjef_N domains and pyridox_oxidase domains. In Prasinophyte algae, only *Ostreococcus tauri* and *Ostreococcus lucimarinus* sequences are available, and no AtPPOX-1 homolog was found.

The AtPPOX-1 homolog derived tree

Figure 3 shows an unrooted neighbor-joining tree of AtPPOX-1 homologs. Clustering of the organisms into fungi, green plants and animals can be observed. However, three clusters were formed for bacteria. AtPPOX-1 homologs of the bacteroidetes form a tight cluster and the other two clusters have bacteria from different families. AtPPOX-1 homologs from plants and animals have much closer relationship than that from fungi. The fungal enzymes are most closely related to AtPPOX-1

homologs found in the genomes of the bacteroidetes. *Cyanidioschyzon merolae* and *Galdieria sulphuraria* are unicellular algae, but they were not in the same cluster. AtPPOX-1 homologs from *Cyanidioschyzon merolae* fall in the same cluster with bacteria, whereas AtPPOX-1 homologs from *Galdieria sulphuraria* fall in the same cluster with green plants. Interestingly, AtPPOX-1 homologs from *Volvox cateri*, the best-known chlorophytes was grouped with animal PPOX.

The AtPPOX-2 homolog derived tree

Figure 4 shows an unrooted phylogenetic tree of AtPPOX-2 homologs. Since animals do not have AtPPOX-2 homolog, only bacteria, fungi and plants are included in this tree. Clustering of the organisms into fungi and land plants can be observed. AtPPOX-2 homologs from cyanobacteria and marine algae such as *Chlamydomonas reinhardtii* and *Ostreococcus sp.*, form a tight cluster, and this cluster is positioned closely to the AtPPOX-2 homologs from land plants. AtPPOX-2 homologs from other bacteria form a different cluster. AtPPOX-1 homologs from fungi show close relation to other bacteria.

DISCUSSION

The subunit of AtPPOX-1 is much longer than that of PPOX found in bacteria, fungi and animals. Besides the C-terminal pyridox_oxidase domain, AtPPOX-1 has an extra N-terminal Yjef_N domain, whereas PPOX in bacteria, fungi and animals have only pyridox_oxidase domain. AtPPOX-1 homologs found in over 30 plant organisms including red algae, land plants, charaphyte algae and chlorophyte algae have both Yjef_N domain and pyridox_oxidase

domain. Yjef_N domain is conserved across all domains of life, but its function is unknown. It exists either as a single protein, or is fused with other domains (Anantharaman and Aravind, 2004). In archea and bacteria, the Yjef_N domain is often found fused to a C-terminal kinase domain of the ribokinase superfamily. In fungi, Yjef_N domain is not only found in a conserved hypothetic protein, but also found in the fungal Dcp3p-like protein. The animal FLJ22/28-like protein contains FDF domain, Sm domain, and Yjef_N domain. The apolipoprotein A-I binding protein that is involved in the regulation of vesicle fusion in the endosomal/lysomomal route in mammals shows a high similarity to the Yjef_N domain family (Schmitz et al., 2004).

In the AtPPOX-1 homolog derived tree, bacteria form three clusters rather than one tight cluster. This demonstrates that AtPPOX-1 homologs from bacteria evolved very rapidly. Fungal AtPPOX-1 homologs are more closely related to bacteroidetes than they are to plants or animals. So the fungal enzyme may have the same origin as bacteroidetes, while enzymes from plants and animals originated from other bacteria. *Cyanidioschyzon merolae* form the same cluster with bacteria, whereas *Galdieria sulphuraria* form the same cluster with green plants. Interestingly, AtPPOX-1 homologs from *Volvox cateri*, the best-known chlorophytes grouped within the animals.

In the AtPPOX-2 homolog derived tree, cyanobacteria and green algae, such as *Chlamydomonas reinhardtii* and *Ostreococcus sp.*, form a tight cluster, and this cluster is closely related to that formed by land plants. Since cyanobacteria are the origin of chloroplast, it is possible that AtPPOX-2 in *Arabidopsis thaliana* was originally present in the endosymbiotic ancestor of the chloroplast, and may target back into the chloroplast after translation.

MATERIAL AND METHODS

Database searches for PPOX and Yjef_N domain family

The non-redundant database of protein sequences was searched using a BLASTP program at NCBI (Altschul et al., 1997), with the Blosum62 matrix and default parameters. We performed PSI-BLAST throughout all organisms using *Arabidopsis thaliana* PPOX (NP_568717 or NP_566081) as the query sequence. Each search resulted in a list of similar sequences, which was added to the next round of PSI-BLAST iteration searches, and each search continued until no new sequences with an alignment score above the default threshold was retrieved. The sequences returned by these queries were combined and all redundant sequences were discarded. Sequences were also retrieved using a tBLASTN search in the EST databases. The returned overlapped EST sequences in the same organism were conjugated into one full EST sequence using the Bioedit program, and then translated into the amino acid sequence. Plant Genomes Central in NCBI was also used to search for EST sequences encoding for PPOX enzymes in plants. PPOX homologs and EST sequences that are not available in NCBI were also retrieved from the DOE Joint Genome Institute. PPOX in *Cyanidioschyzon merolae* was found in http://merolae.biol.s.u-tokyo.ac.jp/blast/blast.html, and EST sequences encoding PPOX in *Galdieria sulphuraria* was found in http://genomics.msu.edu/galdieria/index.html.

Sequence alignments and phylogenetic analysis of PPOX

All protein sequences retrieved using the above methods were examined individually and aligned using Clustal X program (Thompson et al., 1997). Manual adjustments were made where necessary. Cluster analyses were perfomed in Paup* v. 4.0 (Swofford, 2002).

Table 1. AtPPOX-1 homologs. Members of this family are listed in alphabetial order (144 sequences)

Acaryochloris_YP_001518889	Lodderomyces_XP_001525388
Acidiphilium_YP_001233967	Human_NP_060599
Acidovorax_YP_971145	Lottia
Acidovorax_YP_986310	Magnetospirillum_YP_420249
Algoriphagus_ZP_01717977	Mannheimia_EDN74621
Anabaena_YP_321076	Marinobacter_YP_957449
Aquilegia	Medicago
Arabidopsis_NP_568717_5G	Methylibium_A2SJZ5
Ashbya_NP_982729	Methylibium_YP_001022119
Arcobacter_YP_001490460	Methylobacterium_ZP_02118090
Azorhizobium_YP_001524113	Microcystis_CAO90251
Azotobacter_ZP_00418533	Microscilla_ZP_01693022
Bacteroides_NP_810490	Monodelphis_XP_001372689
Bacteroides_YP_098738	Monosiga
Bradyrhizobium_NP_729624	Mouse_NP_598782
Bdellovibrio_NP_969371	Myxococcus_YP_629552
Bordetella_NP_881799	Nectria
Bordetella_Q2KVR0	Neurospora_XP_961897
Botryotinia_XP_001551181	Nodularia_ZP_01632435
Bovine_NP_001014907	Nostoc_NP_485291
Brassica	Nostoc_ZP_00111879
Burkholderia_ZP_01499380	Oceanobacter_ZP_01307236
Caenorhabditis_NP_498518	Opitutaceae_ZP_02013944
Candidatus_YP_266037	Oryza_NP_001064477
Candida_XP_447454	Pedobacter_ZP_01882425
Capitella	Phaeosphaeria_EAT83869
Castor_m006117	Physcomitrella_EDQ52531
Cellulophaga_ZP_01049128	Pichia_XP_001386486
Chaetomium_XP_001223947	Picea
Chloroflexus_ZP_01517186	Picea_ABK24840
Citrus	Pinus
Colwellia_YP_267797	Polaribacter_ZP_01117843
Comamonas_ZP_01518565	Polaromonas_YP_550365
Coxiella_NP_819941	Populus
Croceibacter_ZP_00951035	Porphyromonas_NP_905824
Crocosphaera_ZP_00516266	Prochlorococcus_NP_896023
Cyanidiochyzon_CMM155C	Pseudomonas_ZP_01716544
Cyanothece_ZP_01730885	Psychromonas_ZP_01216352
Cytophaga_YP_678384	Psychroflexus_ZP_01252609
Daphnia	Radish
Danio_XP_688664	Ralstonia
Debaryomyces_XP_461102	Reinekea_ZP_01114160
Deinococcus_YP_603915	Rhodopirellula_NP_869187
Delftia_YP_001564960	Rhodopseudomonas_YP_484826
Dictyostelium_XP_642126	Robiginitalea_ZP_01120734
Dichelobacter_YP_001209911	Roseiflexus_YP_001431186

Drosophila_AAM50875	Rubrobacter_YP_645112
Escherichia_ZP_00712973	Sacchramyces_P38075
Flavobacterium_YP_001296578	Saccharum
Flavobacterium_ZP_01061540	Salinibacter_83815282
Frankia_YP_710403	Salinispora_YP_001157287
Fugu	Selaginella
Galdieria_Gs00430	Schizophyllum_AAC28862
Gibberella_XP_389815	Schizosaccharomyces_NP_594650
Gluconacetobacter	Sclerotinia_XP_001585171
Gluconobacter_YP_192380	Sorangium_YP_001615726
Gossypium_ES822756	Sphingopyxis_YP_615708
Gramella_YP_861872	Stigmatella_ZP_01466924
Haemophilus_ZP_01793932	Synechocystis_NP441623
Hahella_YP_435655	Tenacibaculum_ZP_01052536
Halorhodospira_YP_001003348	Thermosynechococcus_NP_681121
Helobdella	Thiomicrospira_YP_392256
Herminiimonas_YP_001100841	Triticum
Horse_XP_001501967	Vanderwaltozyma_XP_001644128
Hyphomonas_YP_759551	Verminephrobacter_YP_997327
Kluyveromyces_XP_451844	Vibrio_ZP_01866118
Kordia_ZP_02163182	Vitis_CAO61642
Laccaria_EDR13720	Volvox
lentisphaera_ZP_01874593	Xanthobacter_YP_001417377
Leptospira_NP_711347	Xenopus_NP_001086328
Leptospira_YP_798481	Yarrowia_XP_502816
Limnobacter_ZP_01915478	Zea

Table 2. AtPPOX-2 homologs. Members of this family are listed in alphabetial order (56 sequences)

Acaryochloris_YP_001516769	Medicago_AW574109
Alteromonas_ZP_01112037	Methylobacterium_ZP_01850257
Anabaena_YP_322187	Nodularia_ZP_01632312
Ananas_CO731833	Nostoc_NP_488078
Arabidopsis_NP_566081	Oceanobacter_ZP_01306347
Burkholderia_ZP_00983542	Oryza_NP_001048753
Candida_XP_716959	Ostreococcus_CAL56347
Carthamus_EL404654	Ostreococcus_XP_001420885
Centaurea_EH748604	Parvibaculum_YP_001412025
Centaurea_EH787638	Physcomitrella_EDQ64399
Chlamydomonas_XP_001700070	Picea_EX433496
Cichorium_EH680966	Pichia_XP_001484478
Coprinopsis_EAU92424	Polaribacter_ZP_01118414
Crocosphaera_ZP_00516567	Populus_ABK94044
Cucumis_CK085633	Prochlorococcus_YP_001484786
Cyanothece_ZP_01732408	Prochlorococcus_NP_893475
Debaryomyces_XP_458405	Prunus_DY642627
Flavobacteriales_ZP_01108137	Psychroflexus_ZP_01252931
Fragaria_EX662000	Raphanus_EX746271
Fulvimarina_ZP_01439324	Rhizobium_YP_766790
Gossypium_CM028803	Robiginitalea_ZP_01121883
Helianthus_EE645774	Saccharomyces_EDN61616
Laccaria_EDR15974	Synechococcus_YP_475391
Lettuce_DW169932	Trichodesmium_YP_722646
Lodderomyces_XP_001523793	Triticum_CK207357
Lycopersicon_S5289081	Ustilago_XP_759066
Lyngbya_ZP_01621013	Vanderwaltozyma_XP_001644619
Magnetospirillum_ZP_00052487	Vitis_CAO14403

Figure 1. Amino acid sequence alignment between AtPPOX-1 and AtPPOX-2. Amino acids identical in these two proteins are highlighted in black, and conservative substitutions are highlighted in gray. The CLUSTALW program was used for sequence alignment (Thompson et al., 1994).

```
AtPPOX-1    1 MRNVIRRVTTMTFTFLLQSPPLPISPSPPQFSLSSSPLSKTQRFITPSQGSTLTTLCTKVIIPNMQDSGS
AtPPOX-2    1 ----------------------------------------------------------------------

AtPPOX-1   71 PPLSYLTQREAAEIDETLMGPLGFLIDQLMELAGLSVAASIAEVYKPEEYSRVLAICGPGNNGGDGLVAA
AtPPOX-2    1 ----------------------------------------------------------------------

AtPPOX-1  141 RHLHHFGYKPFICYPKRTAKPLYTGLVTQLDSLSVPFVSVEDLPDDLSKDFDVIVDAMFGFSFHGAPRPP
AtPPOX-2    1 ----------------------------------------------------------------------

AtPPOX-1  211 FDDLIRRLVSLQNYEQTLQKHPVIVSVDIPSGWHVEEGDHEDGGIKPDMLVSLTAPKLCAKRFRGPHHFL
AtPPOX-2    1 ---------------------------------------------------------------------M

AtPPOX-1  281 GGRFVPPSVAEKYKLELPSYPGTSMCVRIGKPPKVDISAMRVNYVSPELLEEQVETDPTVQFTKWFDEAV
AtPPOX-2    2 GTHVAP---------------------------------------WKQLLFGAIEAN-------------

AtPPOX-1  351 AAGLRETNAMALSTANKDKKPSSRMVLLKGFDENG--FVWFTNYESKKGSDLSENPSAALLFYWEILNRQ
AtPPOX-2   20 -SHLSHSSYVQLATIGLNGRPSNRTVVFRGFEENSDRIQINTDLRSRKIEELKHCPFSEMCWYFSDTWEQ

AtPPOX-1  419 VRIEGPVERIPESESE-NYFHSRPRGSQIGAIVSKQSSVVPGRHVLYDEYEELTKQYSDGSVIPKPKNWG
AtPPOX-2   89 FRINGRIEVIDASNPDQTKLQQREKAWFANSLRSRLIYVCPTPGSPCNSEQSSQQVKLDPSSGPVP-EYC

AtPPOX-1  488 GFRLKPNLFEFWQGQPSRLHDRLQYS-LQDVNGNPAWKIHRLAP
AtPPOX-2  158 LLLLEPEKVDYLN---LKTNQRLFFSSMATGTGEKCWTSEKVNP
```

Figure 2. Conserved regions among all AtPPOX-1 and AtPPOX-2 homologs

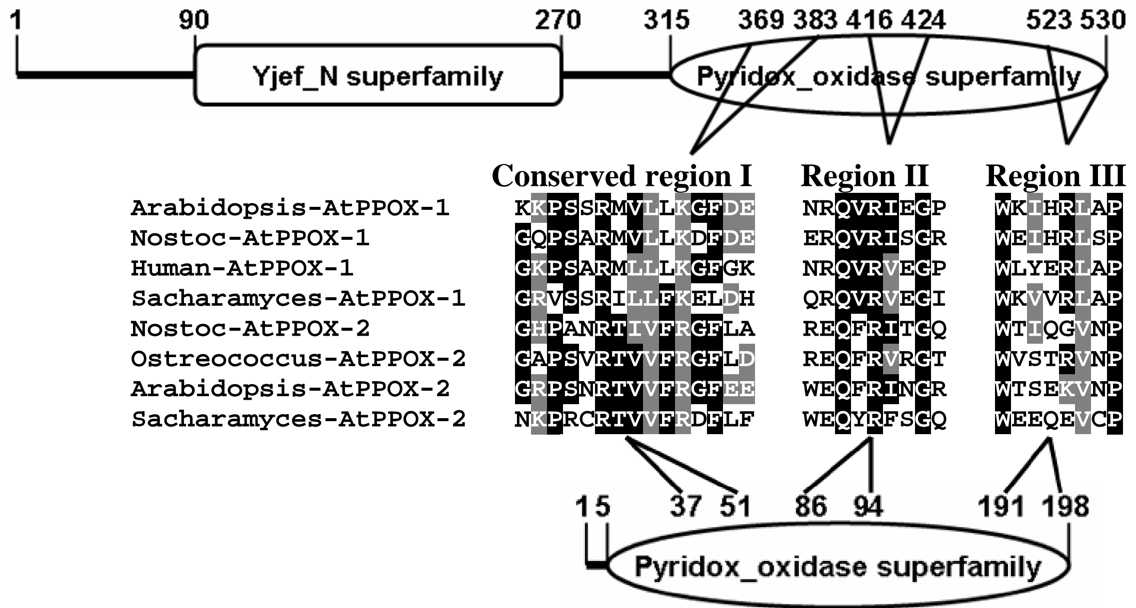


Figure 3. AtPPOX-1 homolog derived tree

Figure 4. AtPPOX-2 homolog derived tree

V EXPRESSIONAL ANALYSIS AND FUNCTIONAL ROLE OF PYRIDOXINE/ PYRIDOXAMINE PHOSPHATE OXIDASE-1 IN *ARABIDOPSIS THALIANA*

ABSTRACT

Pyridoxal phosphate (PLP), a vitamin B_6 vitamer, is an essential cofactor for numerous enzymes. Pyridoxine/pyridoxamine phosphate oxidase (PPOX) catalyzes the synthesis of pyridoxal phosphate from pyridoxine or pyrodoxamine. AtPPOX-1 is one of the PPOX enzymes in *Arabidopsis thaliana* that is involved in the salvage pathway of vitamin B_6 metabolism, and it is responsible for synthesis of PLP. In this study, regulation of the *AtPPOX-1* gene expression and *in vivo* physiological function of AtPPOX-1 were investigated. A variable level of expression of *AtPPOX-1* was seen in all tissues of *A. thaliana* examined. This gene is up-regualted by light, heat shock, ABA, JA and ethylene treatment, and down-regulated by exposure to NaCl. Monoclonal antibodies against different domains of ATPPOX-1 recognized different sizes of protein in root and shoot tissues suggesting expression of alternate splice variants of *AtPPOX-1* in root and shoot. To determine the biological role of AtPPOX-1, T-DNA insertional mutants of *A. thaliana* in *AtPPOX-1* were analyzed. The mutant lines showed sensitivity to NaCl and high light for growth, and sensitivity to high concentrations of sucrose relative to cotyledon development. These results suggest involvement of AtPPOX-1 in stress tolerance and cotyledon development in *A. thaliana*.

INTRODUCTION

Vitamin B_6 is known for its function as an essential cofactor for numerous enzyme reactions (John, 1995). The B_6 vitamers are also excellent antioxidants comparable to vitamin E and C, and are particularly active in quenching singlet oxygen and superoxide anion (Ehrenshaft et al., 1999; Osmani et al., 1999; Jain and Lim, 2001). In plants, vitamin B_6 is involved in stress tolerance, growth and development (Shi et al., 2002; Chen and Xiong, 2005; Studart et al., 2005; Titiz et al., 2006; Wagner et al., 2006; Studart et al., 2007) .

Vitamin B_6 is synthesized in plants using the DXP-independent biosynthesis pathway in which PDX1 and PDX2 proteins are involved (Studart et al., 2005). PDX1 is required for shoot and root development (Chen and Xiong, 2005). In addition to the developmental defects, *PDX1* mutants are hypersensitive to salt, osmotic stress and oxidative stress (Chen and Xiong, 2005). PDX2 is involved in the seed development (Studart et al., 2005; Studart et al., 2007*)*. SOS4 catalyzes the phosphorylation of the vitamin B6 vitamers to form the corresponding phosphorylated derivatives (Lum et al., 2002). An *SOS4* mutant had very short root when treated with high salt (Shi and Zhu, 2002), and *SOS4* mutants do not have root hairs in the maturation zone, suggesting that pyridoxal kinase is required for root hair development in *Arabidopsis thaliana* (Shi and Zhu, 2002). Pyridoxine/pyridoxamine phosphate oxidase (PPOX) is involved in the salvage pathway responsible for synthesis of PLP from PMP or PNP. A PPOX mutant of *Arabidopsis* showed a decrease in root and shoot growth under normal conditions, and was sensitive to high light (Gonzalez et al., 2007).

Regulation of *PDX1* and *PDX2* genes in plants has been extensively studied. *PDX2* and three *PDX1* genes were responsive to abiotic stressors including high light, chilling, drought, and ozone (Denslow et al., 2007). However, the response of *PDX1.2* was different from that of the other *PDX* genes and showed lower sensitivity to high light, chilling, and drought, but an increased sensitivity to ozone (Denslow et al., 2007). *PDX1*and *PDX2* transcript levels in *Nicotiana tabacum* decrease following inoculation with the incompatible pathogen *Pseudomonas syringae* pv. *phaseolicola* and transiently increased in response to salicylic acid and methyl jasmonate (Denslow et al., 2005). However, expression of *PDX1* in *Phaseolus vulgaris* did not change with MeJA treatment. Expression of *pvPDX1* was slightly up-regulated within two hourse of wounding. Treatment with ACC and GA_3 caused the greatest increase in *pvPDX1* expression, while hydrogen peroxide or rose Bengal did not affect *pvPDX1* expression (Graham et al., 2004). *SOS4* expression was modulated by NaCl, abscisic acid, and cold treatment, but not by drought (Shi et al., 2002).

In this study, we describe the expression of the *AtPPOX-1* gene and its regulation by tissue specificity, abiotic stresses and hormones. Results indicat that *AtPPOX-1* gene has two alternative splice variants expressed in different tissues. T-DNA insertional mutants of *AtPPOX-1* in *A. thaliana* were analyzed for their phenotypic properties under different treatments to determine the role of AtPPOX-1 in plant growth and development.

RESULTS

Expression of the *AtPPOX-1* in different tissues of *A. thaliana*

To determine the linear range of amplification during quantitative RT-PCR, different cycle numbers were used (Figure 1). Based on this observation 25 cycles of amplification appeared to be optimal for quantitative RT-PCR. The expression of the *AtPPOX-1* in different tissues of *A. thaliana* was determined, and results are shown in Figure 2. *AtPPOX-1* is highly expressed in leaf and stem at similar levels, but its expression is considerably less in flower. The expression of *AtPPOX-1* in root is higher than that in flower, but lower than that in leaf and stem.

Expression of the AtPPOX-1 protein in different tissues of *A. thaliana*

Expression of AtPPOX-1 was determined by Western blot using anti-PPOXCT monoclonal antibody. Protein from leaf, stem, flower and root tissues demonstrated the presence of PPOX in all tissues in Western blot except in root (figure 2). However, *AtPPOX-1* gene is transcribed in root which also shows PPOX activity. Two sets of SDS-PAGE for shoot and root proteins were independently probed with anti-PPOXCT and anti-PPOXNT monoclonal antibodies in a Western blot analysis. The anti-PPOXNT detected AtPPOX-1 from both shoot and root, while anti-PPOXCT detected only the AtPPOX-1 from shoot. AtPPOX-1 expressed in both shoot and root has a molecular weight of 51 kDa, while the shoot AtPPOX-1 has a molecular weight of 49 kDa (Figure 3A).

AtPPOX-1 protein is not localized in chloroplast

On the basis of bioinformatic analysis, AtPPOX-1 protein was predicted to be localized in the chloroplast according to TargetP (http://www.cbs.dtu.dk/services/TargetP/) and ChloroplastP (http://www.cbs.dtu.dk/services/ChloroP/) servers. To determine if the prediction is correct, intact chloroplast were isolated. Proteins from the intact chloroplast and from the supernatant of chloroplast isolation were blotted using both anti-PPOXCT and anti-PPOXNT. The AtPPOX-1 protein appears not to be present inside chloroplast (Figure 3B). Immunohistochemical analysis of AtPPOX-1 using monoclonal antibodies failed to detect the protein in any compartment of cell (results not shown).

Native PAGE in-gel assay for AtPPOX-1 enzyme activity

In order to determine the number of PPOX proteins in *A. thaliana*, native PAGE in-gel assay was performed. In native basic PAGE in-gel assay, one activity band was observed in leaf, stem and flower tissues, and root (weaker) tissue, and no PPOX activity bands were observed in silique tissue (Figure 4). Western blot using anti-PPOXCT demonstrated that the activity band in the in-gel assay was AtPPOX-1, and no activity band was detected in native acidic PAGE in-gel assays (data not shown).

Regulation of the *AtPPOX-1* gene

The expression of *AtPPOX-1* was regulated by light-dark cycles. Plants grown in dark for 72 hr have a very low expression of *AtPPOX-1*, but expression returned to normal once plants held in the dark were returned to light (Figure 5A). Expression of

AtPPOX-1 was up-regulated by high light, but down-regulated by exposure to 150mM NaCl. No substantial change in expression was noted by exposure to UV and drought treatments (Figure 5B). Heat treatment greatly increased the expression of *AtPPOX-1*. Ethlylene had no effect on the expression of *AtPPOX-1* during an 8 hr exposure. Both ABA and JA treatments resulted in increased expression of *AtPPOX-1* at 4 hr treatment, while expression levels returned to normal after 8 hr treatment (Figure 5C).

Analysis of an *A. thaliana* mutant

Six different T-DNA insertional mutant lines were obtained from ABRC stock center. These lines were self-pollinated for three generations to produce homozygous lines. All of the mutants had significant expression of AtPPOX-1 protein and RNA, and did not show any appreciable phenotypic difference, except that CS825697 with a T-DNA insertion in the promoter region of the gene showed a reduced level of *AtPPOX-1* mRNA.

One-week old seedlings were transferred to MS medium and MS medium supplemented with 100mM sucrose. The phenotype was observed one week after transfer. The CS825697 homozygous mutant showed a difference in growth from wild type (Figure 6). On MS medium with 100mM sucrose, CS825697 had very short cotyledonary petiole. Cotyledon of CS825697 lost function earlier than wild type cotyledon, while CS825697 has stronger true leaves and purple color of shoot which disappeared gradually over time.

One-week old seedlings grown on MS medium and MS medium supplemented with 50mM, 75mM or 100mM NaCl did not show a big influence on the fresh weight of wild

type after two weeks growth, but the fresh weight of CS825697 was significantly increased when grown on MS medium with 50mM and 75mM NaCl, and showed a substantial reduction in fresh weight at 100mM NaCl (Figure 7A). The leaves of CS825697 mutant showed abnormal leaf development and turned yellow (Figure 7B) and had impaired primary root growth, but stronger secondary roots than of the wild type (Figure 7C) on 75mM or higher NaCl plate.

One-week old seedlings were exposed to continuous light in a growth chamber with high light intensity (1000 μmol S^{-1} m^{-2}) for one week. Wild type seedlings continued to grow under high light intensity and fresh weight almost doubled in one week, while the CS825697 mutant stopped growing and fresh weight even decreased after one week exposure to high light intensity (Figure 8).

DISCUSSION

The level of *AtPPOX*-1 expression varied in different tissues of *A. thaliana* with the highest levels of mRNA found in leaf and stem tissues followed by root and flower tissues. Protein levels were consistent with the RNA levels except in the root tissues. Tissue specific expression of other genes/proteins in the vitamin B_6 pathway involving pyridoxal kinase (Lum et at., 2002), *PDX1* and *PDX2* (Wagner et al., 2006; Studart et al., 2007; Titiz et al., 2006) have been shown.

During initial analysis of AtPPOX-1 protein in root and shoot tissues the inconsistency between RNA and protein expression was further analysed. AtPPOX-1 protein in root and shoot tissues showed variation in the size of protein and their differential recognition by ant-PPOXCT and anti-PPOXNT antibodies. SOS4 catalyzes

the pyridoxal kinase reaction in the vitamin B_6 pathway and is regulated by alternative splicing. Two splice variants of *SOS4* are modulated by development and various environmental stresses (Shi et al., 2002). Immunochemical analysis using two different lines of monoclonal antibody against AtPPOX-1 also identified two different AtPPOX-1 proteins: one with a molecular weight of 51 kDa and the other of 49 kDa. Both of them were expressed in shoot, but only the longer transcript was expressed in root. The predicted full length amino acid sequence of AtPPOX-1 has a molecular weight of 59.4 kDa, but the mature protein is 51 kDa in size (Sang et al., 2007). There are two possible explanations for this unexpected result. Either the root or shoot tissues have different splice variants of *AtPPOX-1* RNA that are preferentially translated in respective tissues or the domain of AtPPOX-1 that is recognized by these monoclonal antibodies has undergone post translational modifications. These two alternative possibilities are being examined.

Alternative splicing is an important mechanism for regulating gene expression at the post-transcriptional level and contributes to proteome complexity (Lareau et al., 2004; Reddy, 2007; Stamm et al. 2005). This widespread process comprises various mechanisms such as exon skipping, mutually exclusive exons, intron retention, or the usage of alternative 5' or 3' splice sites (Cartegni et al., 2002). Alternative splicing has been extensively studied in mammals. Recent evidence indicates more than 60% of the genes in the human genome are alternatively spliced (Kim et al., 2006) compared to about 20–30% of plant genes (Campbell et al., 2006; Wang and Brendel, 2006). Although proteins from one gene have the same or similar functions, they can have different

characteristics, such as their binding properties, intracellular localization, enzymatic activity, regulation and stability (Stamm, 2002).

Bioinformatic analysis of AtPPOX-1 suggested that the protein has three domains consisting of Pyridox_oxidase domain at the C terminal, followed by a Yjef_N domain and a putative transit peptide for localization of AtPPOX-1 to chloroplast. Our result using isolated chloroplasts did not support the information produced by bioinformatic analysis. The protein appears to be present in soluble fraction outside the chloroplast. Hence, the function of the N-terminal domain of AtPPOX-1 remains to be determined. Our effort to use immunohistochemical localization of the protein was not successful because the monoclonal antibodies did not recognize the native protein in tissue sections.

Expression of *AtPPOX-1* is reduced in dark and its expression returns to normal when grown under normal light. Expression of *AtPPOX-1* is up-regulated by high light which may induce photoxidative stress. Under high light, wild type grew better than under normal light conditions. However, the growth of AtPPOX-1 insertional mutant line, CS825697 was inhibited. These results suggests a working hypothesis that vitamin B_6 functions as an ROS quenchers. Both *PDX1* and *PDX2* are regulated by light (Titiz et al., 2006; Studart et al., 2007).

When exposed to NaCl, leaves of the CS825697 insertional mutant showed reduced accumulation of chlorophyll. Salt stress inhibits chlorophyll accumulation partly by reducing the rate of porphyrin formation but also by a possible reduction in the formation of chlorophyll-binding proteins (Abdelkader et al., 2007). The high salt-induced reduction of chlorophyll content is mediated by decreased synthesis of 5-aminolaevulinic acid, a

precursor of chlorophyll, rather than the chlorophyllase mediated degradation of chlorophyll (Conceicao, 2004).

The T-DNA insertional mutant of AtPPOX-1, CS825697, shows altered development of cotyledons when grown on MS media containing high concentrations of sucrose, but not in low concentrations of sucrose or in other treatments. The result indicated that the alteration of petiole development is mediated by sucrose sensing, which suggests that AtPPOX-1 or PLP may be a component of the sucrose-signaling pathway or a sucrose sensing pathways.

All of the insertional mutants of *A. thaliana* had substantial AtPPOX-1 protein. Our effort to obtain a highly reduced level of AtPPOX-1 by RNAi silencing did not yield any transgenic lines with substantial reduction in AtPPOX-1. Transgenic lines overexpressing AtPPOX-1 under the control of a strong promoter produced slightly higher levels of mRNA but there was no significant difference in the level of AtPPOX-1 protein (data not shown). All these results suggest that a minimal level of expression of AtPPOX-1 is required for survival of *A. thaliana*, and that the level of this protein expression is highly regulated.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis seeds were first placed at 4ºC for at least 2 days to break the dormancy of the seeds, and then were sown by sprinkling them onto the damp soil covered with a transparent plastic cover. The cover was removed after seeds germinated. Two-week-old seedlings in the growth chamber at 22ºC with continuous light were then transferred into

individual pots for stress treatment. When using solid media, seeds were surface sterilized, rinsed with sterile water and placed in rows onto Murashige and Skoog (MS) nutrients agar media (MP Biomedicals, Ohio). After exposure of 4ºC for 2 days, the plates were placed vertically in the growth chamber at 22ºC with continuous light. One-week old seedlings were then transferred to MS plates with or without treatment.

Stress treatments for quantitative RT-PCR

For dark treatment**,** four-week old Arabidopsis seedlings grown on soil were moved to a dark chamber for different length of time. Some of the plants were moved back to the light after dark treatments. For high light treatment, four-weeks old seedlings were moved to an 8 hr photoperiod at 1000 μmol S^{-1} m^{-2} light. For UV light treatment, seedlings were moved to a UV box for 1 hr treatment at 30uJ/m·S UV light. For drought stress treatments, seedlings growing in the soil were not watered until they became wady. For NaCl treatment, seedlings were irrigated with 150mM NaCl for one week. For heat treatment, seedlings were transferred to 42ºC for different times as indicated in text, table and figure legends. For ABA and JA treatment, seedlings were sprayed with 100µM ABA or 200 µM JA and grown as indicated n text table and figure legends. For ethylene treatment, the seedlings were placed in a chamber with 100ppm ethylene.

Isolation of RNA and quantitative RT-PCR

A. thaliana seedlings were ground to a powder in liquid nitrogen. RNA was extracted using RNAeasy plant mini kit (Qiagen), as described in the instruction manual. Total RNA (0.4 µg) was reverse-transcribed using the one-step RT-PCR kit (Qiagen) in a

50 μl RT-PCR reaction mixture. Amplification was performed in a thermal-cycler as follows: 30 min at 50°C; 15 min at 95°C; then 1 min at 94°C; 1 min at 55°C; 1 and a half min at 72°C for 25 cycles, followed by 10 min at 72°C. CBP20 primers were added together with AtPPOX-1 primers into the reaction mixture as internal control. Primers for AtPPOX-1 were 5'- ATGAGGAATGTGATACGCAGAGTC-3' and 5'-TCATGGGGCCAATCTATGAA -3'. Primers for CBP20 were 5'-ATGGCTTCT TTGTT CAAGGAGC-3' and 5' –TTAAGATCTTCTCTTCCGATCATC -3'.

Chloroplast isolation

Chloroplasts were isolated with some modification described by Locy et al., 1996. Fresh leaf tissues of *A. thaliana* were suspended in chilled grinding buffer (50 mM Hepes-KOH, pH 8.3, 350 mM Sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM EGTA, 0.5% BSA, 4.4 mM ascorbate), and were homogenized in a Waring Blendor equipped with razor blades at the top speed three times for 5 seconds each. The homogenate was filtered through two layers of Miracloth (Calbiochem) between two layers of cheesecloth. The filtrate was centrifuged for 5 min at 3000 rpm in a Sorvall HB-4 rotor to pellet the crude chloroplast fraction. The pellets were gently resuspended in grinding buffer using a soft brush. The suspension was layered onto a percoll step gradient consisting of 40 and 80% (v/v) percoll. The gradients were centrifuged at 9000 rpm for 4 min at 4ºC in a Sorvall HB-4 rotor with the brake turned off. The upper band containing broken membranes was separated from the middle band containing intact chloroplasts. The middle band was diluted with three times the volume of grinding buffer, and repelletted by centrifuge. The pellet was resuspended in the grinding buffer, and then

reloaded onto a second percoll step gradient. Resuspension buffer (50 mM Hepes-KOH, pH 8.3, 375 mM Sorbitol, 10 mM Na_2HPO_4, 0.96 mM DTT) was added to the material from the middle band, and after gentle mixing, the mixture was centrifuged at 4000 rpm for 4 min to wash of the percoll. The pellet was dissolved in the SDS-PAGE loading buffer or saved at -80ºC for future use.

Native PAGE in-gel enzyme assay for Pyridoxine/Pyridoxamine 5'-Phosphate Oxidase

Basic native PAGE gel was carried out using 7.5% acrylamide separating gel (pH 8.8), 3% acrylamide stacking gel 6.8%, and Tris-glycine (pH8.3) as the running buffer. After loading samples, the electrophoresis was carried out at 4ºC, and 10 mA current was applied. PPOX enzyme activity was visualized by incubating the gels in 0.2M Tris-HCl (pH 8), 1.9mM PNP, 0.1 mg/ml of nitroblue tetrazolium, 0.05mg/ml of phenazine methosulfate, and 1 μM FMN at room temperature for 5 hours until maroon bands appeared (Kazarinoff and McCormick, 1975).

Preparation of monoclonal antibody against PPOXCT and PPOXNT

The recombinant proteins derived from Yjef_N domain of AtPPOX-1 (PPOXNT) and the Pyridox_oxidase domain of AtPPOX-1 (PPOXCT) were expressed and purified using the method described by Sang et al (2007). The purified recombinant proteins were treated with enterokinaseMax (Invitrogen) to remove the C-terminal 6×His and Xpress™ tag following the manufacturer's instructions, and separated on the SDS-PAGE. The respective proteins were eluted from gel after electrophoresis using a Bio-Rad Hercules

whole-gel elutor (Bio-Rad). The eluted proteins were dialyzed against double-distilled water at 4ºC and quantified with the Bradford microassay with BSA as the standard (Bradford, 1976). The monoclonal antibodies against PPOXNT and PPOXCT were generated in mice at Hybridoma Facility at Auburn University, AL.

Immunochemical analysis

For Western Blot analysis, proteins were extracted from fresh plant tissue by homogenization in liquid nitrogen and 1 volume of 50 mM Tris-HCl, pH7.5, containing 100 mM NaCl and 0.5% NP-40. Debris was removed by centrifugation at 10,000 g for 15 min at 4ºC. Total protein was quantified using Bradford method, and was separated on 12.5% SDS-PAGE. For immunodetection, the protein was blotted onto a nitrocellulose membrane and blocked by incubation with Tris buffered saline (TBS; 10mM Tris-HCl, pH7.5, 0.9% NaCl) containing 0.05% Tween-20 and 5% milk powder. AtPPOX-1 was detected by incubating the blots for 1 hr with monoclonal anti-PPOXCT and anti-PPOXNT. This was followed by incubation of 30 min with a 1:5000 dilution of a donkey anti-mouse alkaline phosphatase conjugated secondary antibody. NBT and BCIP substrates were used for the detection of the blot.

T-DNA insertional mutants

Five lines of T-DNA insertional mutants were obtained from ABRC stock Center, Ohio State University. The mutant lines were self-pollinated for three generations to obtain homozygous lines that did not show further segregation. Total RNA was extracted from the mutant seedlings, and quantitative RT-PCR was used to determine the level of

AtPPOX-1 expression in each of the mutant lines. Level of AtPPOX-1 protein in these mutants was determined by Western blot using anti-PPOXCT.

Seeds from the mutant lines and wild type were surface sterilized as above and plated on MS medium with 1.2% agar and pH 5.7. Plates were placed at 4 ºC for 48 hr to synchronize germination and then incubated vertically at 22 ºC under continuous light. One-week old seedlings were transferred to MS medium alone or supplemented with 100mM NaCl, 100mM Mannitol, 100mM Sucrose. Response of the mutant lines and wild type to high light was carried out at continous1000 µmol S^{-1} m^{-2} light. Seedlings were monitored for phenotypic changes for 2 weeks, after which all plants were harvested and fresh weighs were determined.

Figure 1. Determination of the optimal amplification cycle during RT-PCR. Three RT-PCR reactions were retrieved from the thermocycler at the indicated cycles and analyzed on 1% agarose gel electrophoresis.

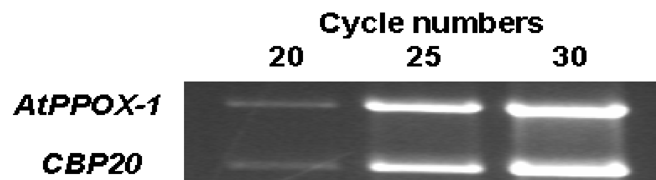


Figure 2. Expression of AtPPOX-1 in different tissues of *A. thaliana*. The level of *AtPPOX-1* RNA in different tissues was determined by quantitative RT-PCR and the result were normalized using internal, CBP20 RNA level in each reaction. Anti-PPOXCT antibody was used for determination of AtPPOX-1 protein in different tissues.

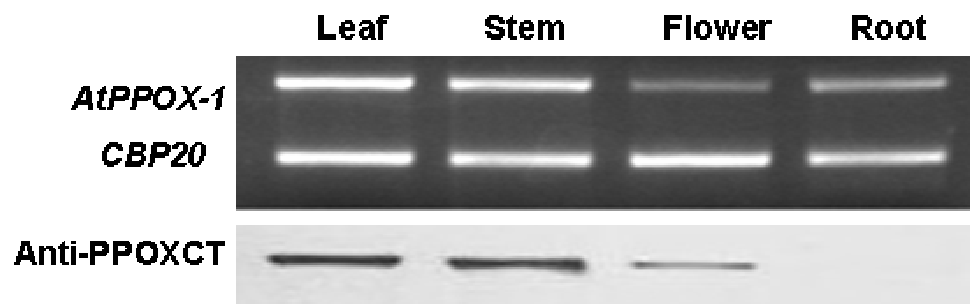
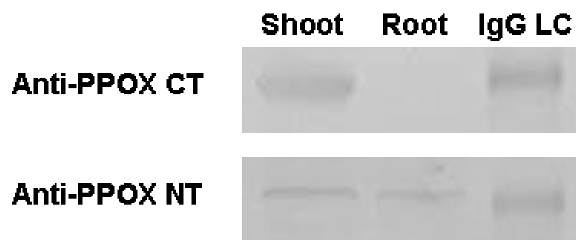


Figure 3. Different forms of AtPPOX-1 in root and shoot tissues (A) and localization of AtPPOX-1 protein (B). Total protein from shoot and root were separated on a 12.5% SDS-PAGE, transferred onto a nitrocellulose membrane, and then probed with anti-PPOXCT and anti-PPOXNT respectively. Total RNA from shoot and root amplified using RT-PCR and were separated on a 2% agarose gel. B) Proteins from the intact chloroplast and soluble supernatant outside intact chloroplast were analyzed by Western blot using mixture of anti-PPOXCT and anti-PPOXNT monoclonal antibodies.

A)



B)

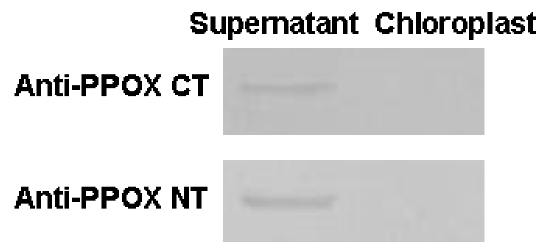
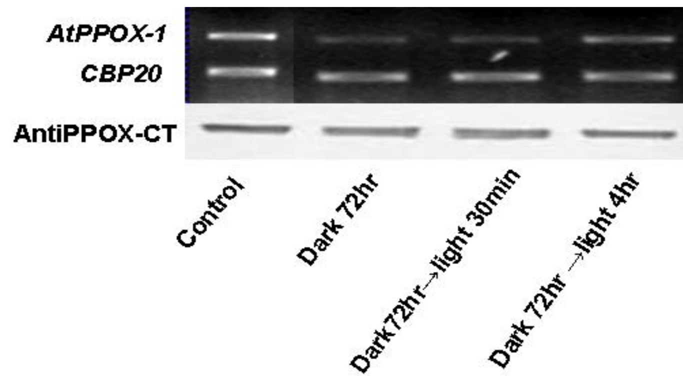


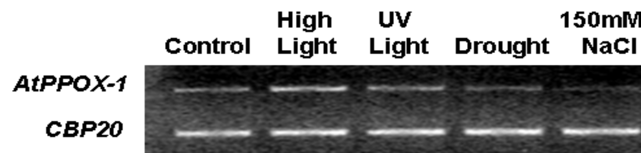
Figure 4. Native basic PAGE in-gel assay for AtPPOX-1 enzyme activity. 144, 115, 204, 15, 44 μg protein was loaded for leaf, stem, flower, silique, and root tissue respectively.

Figure 5. Regulation of *AtPPOX-1* by environmental and hormone factors. A) Analysis of AtPPOX-1 expression in light-dark cycles. Control is the plants growing at 25 ºC with continuous light. B) Analysis of AtPPOX-1 expression with high light, UV light, drought and NaCl treatment. C) Analysis of AtPPOX-1 expression with heat, ABA, JA and ethylene treatment.

A)



B)



C)

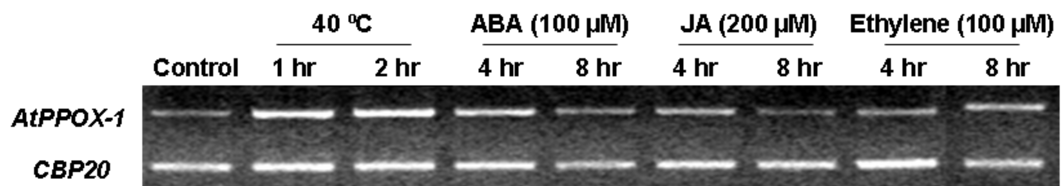


Figure 6. Two-week old wild type and CS825697 seedlings grown on MS agar plate with or without 100 mM sucrose. Phenotypic differences were recorded two weeks after transfer.

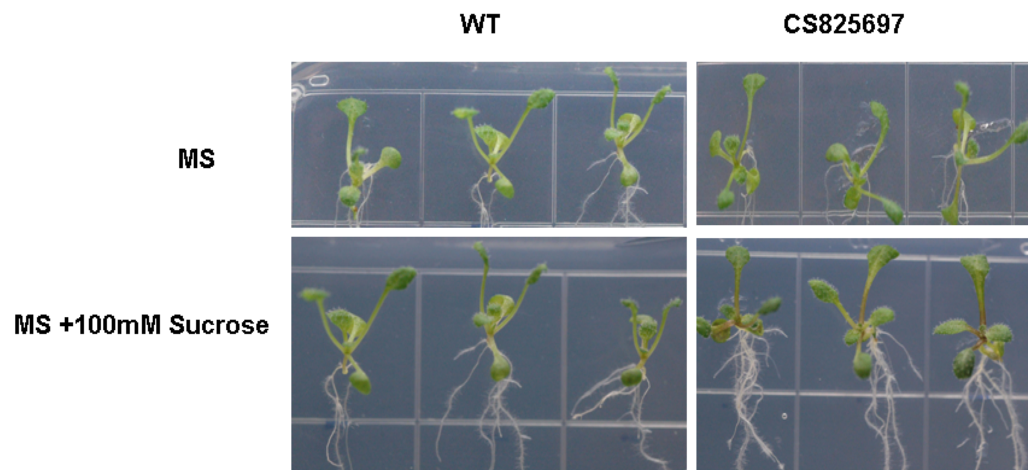
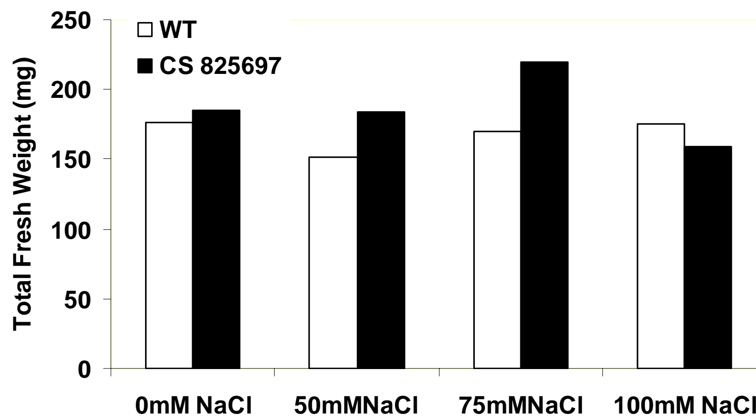
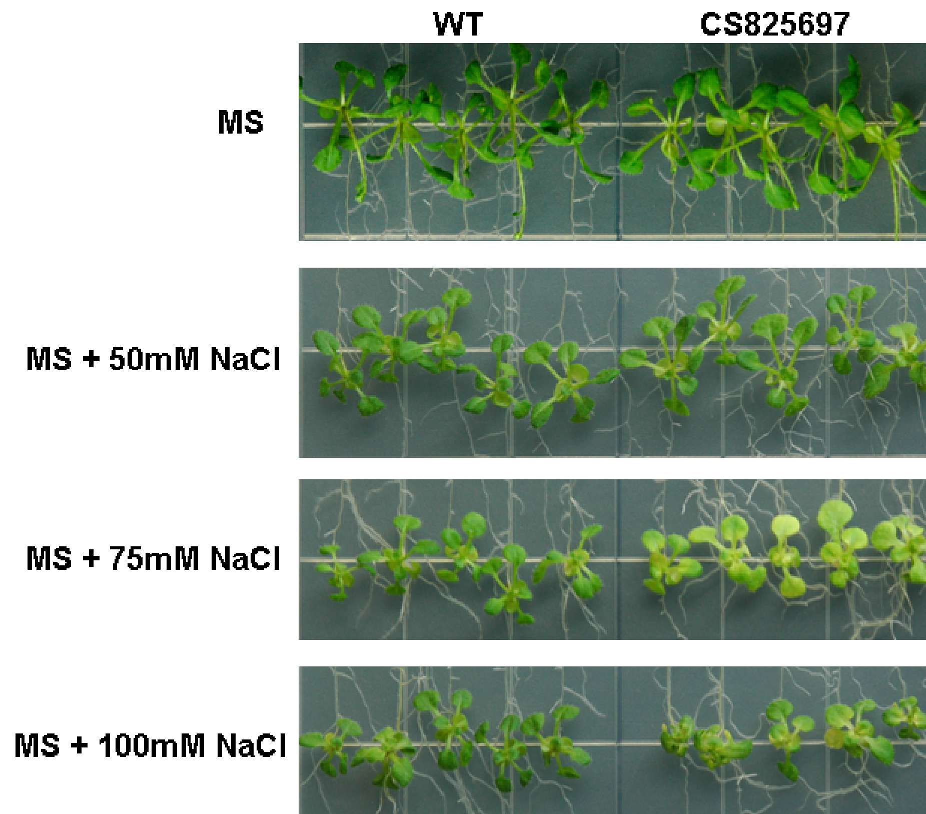


Figure 7. Sensitivity of CS825697 to NaCl. Morphology of wild type and CS825697 mutants grown on vertically placed MS agar plates supplemented with different concentrations of NaCl. Seedlings were first grown on vertical MS agar plates for seven days before being transferred to vertical agar plates without or with NaCl. The plates were placed upside down for root bending. Fresh weight and the photographs were taken 2 weeks after the transfer. Fresh weight of WT and CS825697 plants grown on MS plate supplemented with different concentrations of NaCl (A); Response of the leaves of CS825697 mutants to NaCl (B); Root growth of WT and CS825697 plants grown on MS plate supplemented with 50mM NaCl (C).

A)



B)



C)

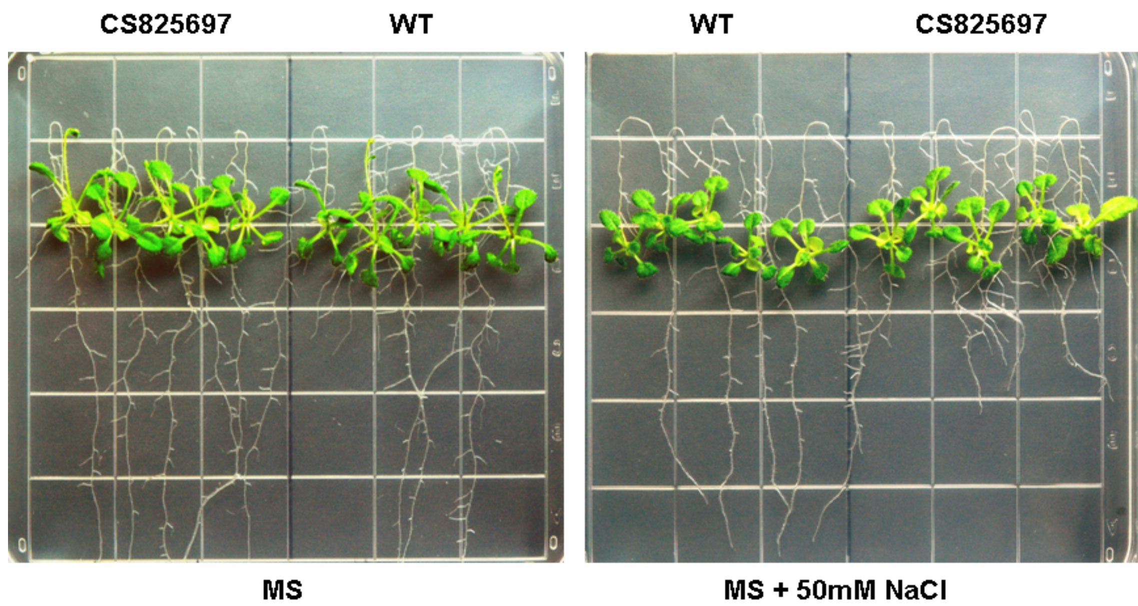
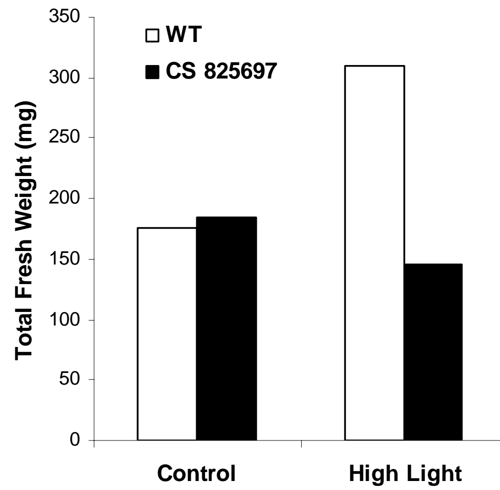


Figure 8. Response of wild type and CS825697 to high light exposure. One-week old seedlings were transferred to MS medium plate and placed in a chamber with high light. Fresh weight was obtained one week after transfer.



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