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Identification of a pyridoxine (pyridoxamine) 5′-phosphate oxidase from *Arabidopsis thaliana*

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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 cerevisiae PDX3* gene. A cDNA corresponding to putative *A. thaliana* PPOX (*At*PPOX) was obtained using reverse transcriptase-polymerase chain reaction 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 deficient in PPOX activity exhibited sensitivity to oxidative stress. Constructs of *AtPPOX* cDNA of different lengths complemented the *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.

Keywords: Pyridoxine (pyridoxamine) 5′-phosphate oxidase; Arabidopsis thaliana; PDX3 knockout yeast; Yjef_N domain; Pyridox_oxidase domain

1. 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 must either produce PLP 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 [1]. Additionally, PLP or its derivatives may function as regulatory molecules in signal transduction, regulating a number of membrane ion transporters [2–4]. Vitamin B6 is also an efficient singlet oxygen quencher and potential antioxidant [5]. PLP is required for post-embryonic root development, and protects plants from high-salt, ultraviolet rays, osmotic and oxidative stresses [6,7].

Most bacteria, fungi, and plants possess vitamin B6 biosynthesis pathways, but mammals must be supplied the vitamin in their diet [8]. Different vitamin B6 biosynthetic pathways, referred to as de novo biosynthetic pathways, and the salvage pathway, are known. In *E. coli*, PNP is synthesized de novo from the condensation of deoxyxylulose 5-phosphate and 4-hydroxythreonine-4-phosphate, catalyzed by PdxA and PdxJ [9]. A number of other bacteria, plants, and fungi utilize ribose 5-phosphate or ribulose 5-phosphate and dihydroxyacetone phosphate or glyceraldehyde 3-phosphate to synthesize PLP [1,10].

The vitamin B6 salvage pathway is involved in interconversions between different B6 vitamers. In vitamin B6 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 B6 phosphatase are all involved in the salvage pathway of plants [8]. However, in this pathway only pyridoxal kinase has been studied in detail [6,11]. This enzyme catalyzes the transfer of a phosphate from ATP to vitamin B6 vitamers resulting in the corresponding phosphorylated derivatives [12]. PPOX plays an important role in the salvage pathway because it converts PMP or PNP to PLP, the active form of vitamin B6 [13]. PPOX has been identified in *S. cerevisiae*, human, mammalian cells, *E. coli*, and several other bacteria [14–17], but little information is available about PPOX in plants. Two isozymes of PPOX from wheat seedlings were partially purified. One isoenzyme used only pyridoxine 5′-phosphate as substrate, while the other one used both PNP and PMP with approximately equal efficiency [18].

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.

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Abbreviations: PPOX, pyridoxine (pyridoxamine) 5′-phosphate oxidase; *At*PPOX, *A. thaliana* pyridoxine (pyridoxamine) 5′-phosphate oxidase; RT-PCR, reverse transcriptase-polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; YPD, yeast, peptone, dextrose medium

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2. Materials and methods

2.1. RNA extraction and cDNA cloning

Two-week-old *A. thaliana* seedlings were ground to a powder in liquid nitrogen. RNA was extracted using the RNAeasy plant mini kit (Qiagen), as described by the manufacturer. Total RNA (0.5 μg) was reverse-transcribed using the one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Qiagen) in a 50 μl RT-PCR 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; 1.5 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.

2.2. Expression of recombinant protein and purification of the AtPPOX

A set of primers (5′-ATGCAAGATTCAGGATCACCAC-3′ and 5′-TCATGGGGCCAATCTATGAA-3′) was used to amplify the *AtPPOX* without the putative transit peptide sequence. The resulting PCR product was 1401 bp long. Another pair of primers (5′-ATGCAAGATTCAGGATCACCAC-3′ and 5′- GTCGACTTAAATTCTAACACACATAGATGTCC-3′) was used to amplify the C-terminal pyridox-domain of the *At*PPOX 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 Xpress™ 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 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 h at 37 °C. The recombinant proteins were purified using the ProBond™ purification System (Invitrogen) under non-denaturing conditions as described by the manufacturer.

2.3. Assay of AtPPOX enzyme activity

PMP was obtained from Sigma Chemical Co. PNP was synthesized by reducing PLP with sodium borohydride [19]. PNP and PMP oxidase activities were measured by monitoring PLP formation in Tris-phosphate buffer as described by Zhao and Winkler [20]. At 414 nm, the Schiff base formed between Tris and PLP has an extinction

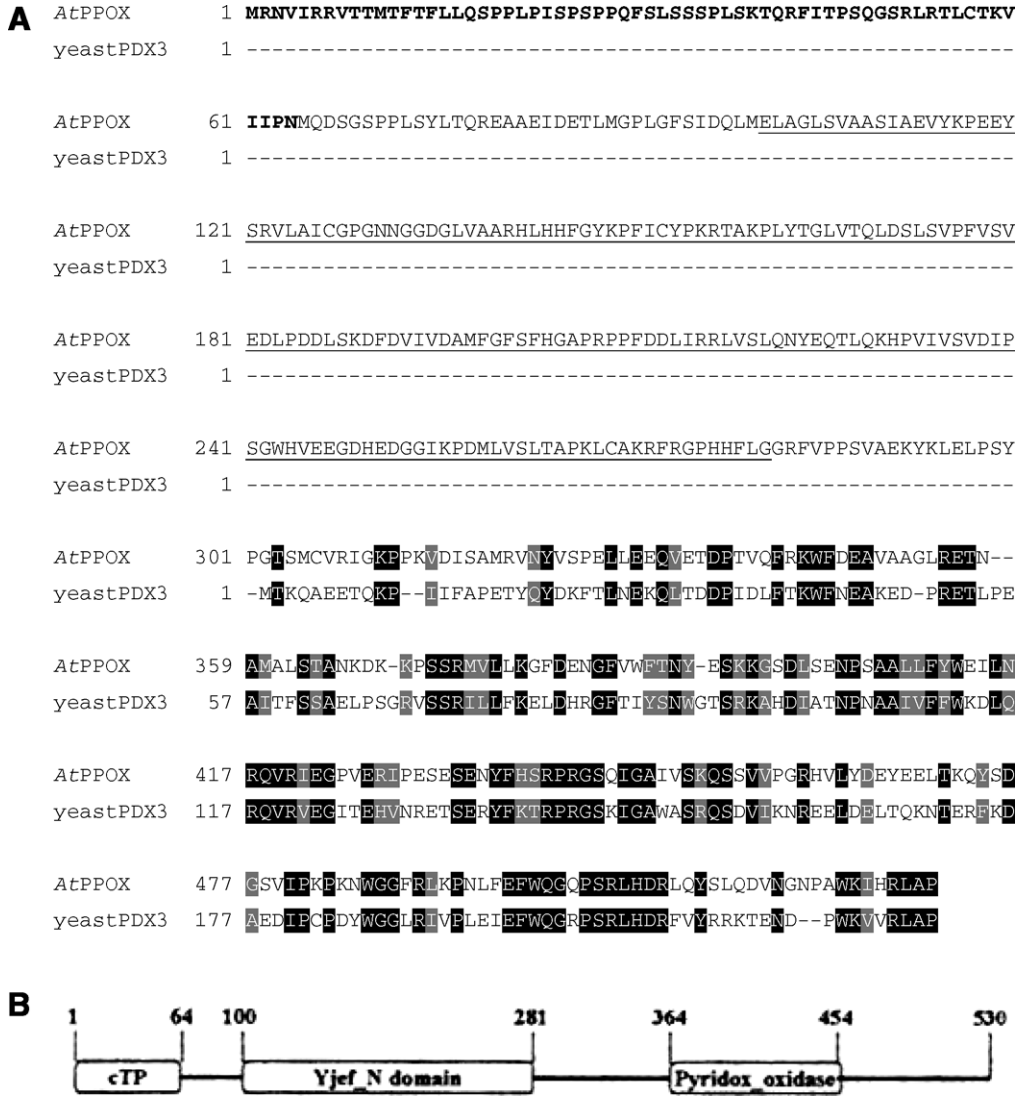

Fig. 1. (A) Amino acid sequence alignment of *At*PPOX and yeast PDX3. Amino acids identical in these two proteins are highlighted in black, and conservative substitutions are highlighted in gray. The putative chloroplast transit peptide is shown in bold and the Yjef_N domain is underlined. The CLUSTALW program [23] was used for sequence alignment. (B) Schematic diagram of predicted domains of *At*PPOX. *At*PPOX contains a putative chloroplast transit peptide, an N-terminal YjeF_N domain, and a C-terminal Pyridox_oxidase domain.