ELSEVIER

Physiological and Molecular Plant Pathology 66 (2005) 244–255

PMPP

www.elsevier.com/locate/pmpp

Regulation of biosynthetic genes and antioxidant properties of vitamin B_6 vitamers during plant defense responses

Sheri A. Denslow [a], Amanda A. Walls [b], Margaret E. Daub [b,*]

[a] *Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695-7616, USA*
[b] *Department of Botany, North Carolina State University, Campus Box 7612, 2214 Gardner Hall, Raleigh, NC 27695-7612, USA*

Accepted 10 September 2005

Abstract

Vitamin B_6, an essential cofactor in enzymatic reactions, has only recently been linked to cellular oxidative stress. We investigated the role of this vitamin as an antioxidant in oxidative responses linked to plant defense. B_6 vitamers effectively quenched superoxide and had antioxidant activity when assayed in vitro. The de novo B_6 biosynthetic genes (*PDX*1 and *PDX*2) were identified in *Nicotiana tabacum* cv. 'Burley 21' and their transcript abundance was assayed during defense responses. *PDX1 and PDX2* transcript levels decreased following inoculation with the incompatible pathogen *Pseudomonas syringae* pv. *phaseolicola* and transiently increased in response to salicylic acid and methyl jasmonate. Excess vitamin B_6 in tobacco leaves interfered with the development of a hypersensitive response caused by *P. syringae* pv. *phaseolicola* and increased disease severity caused by the compatible bacterium *P. syringae* pv. *tabaci*. Our findings indicate that during plant defense responses, vitamin B_6 functions and its synthesis is regulated in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species in vivo.

Keywords: Vitamin B_6; Pyridoxine; Antioxidant; Plant defense; *PDX1*; *PDX2*; *SOR1*; *SNO*; *SNZ*; *Nicotiana tabacum* cv. 'Burley *21*'; *Pseudomonas syringae pv. phaseolicola*; *Pseudomonas syringae* pv. *tabaci*

1. Introduction

Vitamin B_6, the collective name given to pyridoxine, pyridoxamine, pyridoxal and their phosphorylated derivatives, is an essential cofactor for numerous enzymatic reactions. It is most notable for its contribution to amino acid biosynthesis where it serves as a cofactor for enzymes involved in decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions [24,48]. Other significant functions include its involvement in carbohydrate and lipid metabolism, in producing some antibiotic precursors, and in synthesizing aminocyclopropane-1-carboxylate (ACC) [24,48].

The vitamin B_6 pathway is poorly characterized in plants. Vitamin B_6 biosynthesis has been thoroughly characterized in *Escherichia coli* and involves a de novo pathway that produces pyridoxine 5′-phosphate as well as a salvage pathway that interconverts between the different vitamers [4,18,24,32–34,41–43,46,52,54,63]. Our lab and others have recently documented that plants, fungi, archaebacteria, and most eubacteria use a distinct de novo biosynthetic pathway involving two genes, *PDX*1 and *PDX*2, that have no homology to the *E. coli* de novo biosynthetic genes *pdxA* and *pdxJ* [25,26,48]. To date, little is known about the enzymes encoded by these genes or the substrates in this pathway. The *PDX*2 gene product has recently been shown to be a glutaminase [6,21], and is hypothesized to be involved in production of the nitrogen-containing substrate for the PDX1 protein. It is known that *PDX1 and PDX2* proteins form a complex [21], but the PDX1 sequence provides no clues to its function. We recently demonstrated that *PDX*1 complements *pdxJ*, and to a more limited extent *pdxA*, mutations in *E. coli*, strongly suggesting that PDX1 catalyzes the ring closure reaction of the pyridoxine molecule [60].

The importance of vitamin B_6 as a cofactor is well established, but it is only in the last decade that research has linked vitamin B_6, often unknowingly, to oxidative stress. The earliest suggestions of a connection between vitamin B_6 and cellular antioxidant defense were based on regulation studies of homologues of *PDX*1 and *PDX*2 [25,26]. In yeast (*Saccharomyces cerevisiae*), the *PDX*1 and *PDX*2 homologues, *SNZ*1

* Corresponding author. Tel.: +1 919 513 3807; fax: +1 919 515 3436.
E-mail address: margaret_daub@ncsu.edu (M.E. Daub).

0885-5765/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pmpp.2005.09.004

and *SNO*1, show increased transcript and protein accumulation at entry into stationary phase, a time of high oxidative stress [11,50]. In the bacterium *Bacillus subtilis*, there is increased protein accumulation of the *PDX*1 homologue upon treatment with paraquat, an inducer of superoxide [3]. H_2O_2 treatment of *Schizosaccharomyces pombe* leads to increased transcript accumulation of the *PDX*2 homologue as well as pyridoxal reductase, a gene whose product is part of the B_6 salvage pathway [13]. Pyridoxal kinase, another component of the salvage pathway, has been connected to salt tolerance and cold response in *Arabidopsis thaliana* [55], and one of the *Arabidopsis PDX*1 homologues (on chromosome 5) shows increased transcript accumulation after exposure to UV-B radiation [12].

In addition to gene regulation studies, metabolic evidence has also been mounting that vitamin B_6 is an essential antioxidant and a strong quencher of active oxygen species. In previous work, we showed that vitamin B_6 is a potent quencher of singlet oxygen, with quenching rates comparable to or greater than those of vitamins C and E, two of the most efficient biological antioxidants known [7]. Vitamin B_6 biosynthetic genes are necessary for resistance to singlet oxygen-generating photosensitizers in the fungus *Cercospora nicotianae* [27]. Also, a recent study in *Arabidopsis* showed that vitamin B_6 decreases singlet oxygen-induced death in *flu* mutant protoplasts [14]. The antioxidant activity of vitamin B_6 is corroborated by findings from animal models. In blood assays, vitamin B_6 had three times the antioxidant activity of vitamin C [57] and was shown to quench superoxide production [35,37]. Vitamin B_6 prevented protein oxidation in rabbit lens cells (a cause of cataract formation) [36], and reversed the elevated inflammatory response and lipid peroxidation characteristic of vitamin B_6-deficient rats [40]. Clinical trials have shown that vitamin B_6 supplements prevent or delay eye and nerve damage associated with diabetes and attributed to superoxide production [35].

In 1995, a report was published showing that treatment of rubber tree (*Hevea brasiliensis*) with salicylic acid and ethephon, chemical inducers of plant-pathogen defense responses, boosted transcript accumulation of what are now known to be *Hevea* homologues of *PDX*1 [56], suggesting that vitamin B_6 may be important during plant defense responses. During pathogen attack, plants produce active oxygen species (AOS) such as superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide. AOS play a central role in plant defenses to pathogen attack, acting as antimicrobial agents, as substrates in cell wall fortification, and as signaling molecules for the activation of defense pathways [5,8,15,20,29,44,45,47]. AOS production must be tightly controlled as over-accumulation of AOS can result in unwanted cell death. Thus, plants must sustain AOS levels at the site of infection to ward off pathogens and to stimulate the defense response, yet keep AOS levels lower in regions not affected by the pathogen to maintain tissue integrity and viability. To help with this fine-tuning, plants employ AOS scavenging enzymes, such as ascorbate peroxidase and catalase, and metabolites including glutathione, ascorbic acid, and α-tocopherol, during pathogen defense [49].

Since the study on *Hevea PDX*1 gene expression, no further studies on vitamin B_6 and plant defense responses have been published. We were interested in defining the possible role of vitamin B_6 in plant defense responses. In addition, the requirement for AOS for successful plant defense suggested to us that the hypersensitive response (HR) may provide an ideal system to confirm the in vivo activity of vitamin B_6 as an antioxidant. Here, we report on the ability of B_6 vitamers to quench superoxide and prevent lipid peroxidation, the regulation of B_6-synthesizing genes following salicylic acid and methyl jasmonate treatment and pathogen inoculation, and the effect of elevated levels of pyridoxine on the course of defense responses in tobacco. Our results show that vitamin B_6 can act as an antioxidant in planta, and that vitamin B_6 biosynthetic genes are regulated during plant-pathogen defense responses in a manner consistent with this vitamin's activity as an antioxidant and modulator of active oxygen species in vivo.

2. Materials and methods

2.1. Superoxide quenching assay

B_6 vitamers, pyridoxine, pyridoxal, and pyridoxamine, were tested for their ability to quench superoxide generated via a xanthine–xanthine oxidase reaction and monitored through the superoxide-mediated reduction of cytochrome *c*. The reaction mixture contained a final concentration of 50 mM KH_2PO_4, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 mM cytochrome *c* (Sigma, St Louis, MO), and 0.05 mM xanthine (Sigma) at pH 7.8. Pyridoxine, pyridoxal and pyridoxamine (Sigma) were added to this solution at final concentrations of 0.1, 1, 10 and 50 mM, and solutions were re-adjusted to a pH of 7.8. Xanthine oxidase (0.0025 units) (Sigma) was added immediately before measurement. All reactions were carried out at 25 °C in a total volume of 1.5 mL. The reaction was measured at 15 s intervals for 5 min using a Beckman DU 650 spectrophotometer to measure A_{550} against a blank lacking xanthine oxidase. Percent inhibition was calculated by the following equation based on a linear slope:

$$\%\ \text{inhibition} = \frac{\text{slope uninhibited} - \text{slope inhibited}}{\text{slope uninhibited} - \text{slope blank}} \times 100$$

Superoxide dismutase (SOD) (Sigma) was used as a control for the assay. In our assay, 0.25 μg SOD (defined by the manufacturer Sigma as equal to one unit) in a final reaction volume of 1.5 ml, resulted in approximately 50% inhibition. Each assay was repeated three times. Statistical differences were determined using a *t*-test.

2.2. Lipid peroxide quenching assay

The antioxidant activity of B_6 vitamers, pyridoxine, pyridoxal, and pyridoxamine, was tested in a spectrophotometric assay measuring inhibition of the coupled oxidation of β-carotene and linoleic acid as described by Hammerschmidt and Pratt [31] and Daub [16]. The reaction mixture contained