Plant Physiology and Biochemistry 85 (2014) 9–13

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Enzymatic conversion from pyridoxal to pyridoxine caused by microorganisms within tobacco phyllosphere

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ARTICLE INFO

Article history:
Received 25 May 2014
Accepted 9 October 2014
Available online 11 October 2014

Keywords:
Nicotiana tabacum
Phyllosphere microorganism
Pyridoxal
Enzymatic reduction
Vitamin B_6

ABSTRACT

Vitamin B_6 (VB_6) comprises six interconvertible pyridine compounds (vitamers), among which pyridoxal 5′-phosphate (PLP) is a coenzyme involved in a high diversity of biochemical reactions. In plants, PLP is *de novo* synthesized, and pyridoxine (PN) is usually maintained as the predominant B_6 vitamer. Although the conversion from pyridoxal (PL) to PN catalyzed by PL reductase in plants has been confirmed, the enzyme itself remains largely unknown. We previously found pre-incubation at 35 °C dramatically enhanced PL reductase activity in tobacco leaf homogenate. In this study, we demonstrated that the increase in the reductase activity was a consequence of phyllosphere microbial proliferation. VB_6 was detected from tobacco phyllosphere, and PL level was the highest among three non-phosphorylated B_6 vitamers. When the sterile tobacco rich in PL were kept in an open, warm and humid environment to promote microorganism proliferation, a significant change from PL to PN was observed. Our results suggest that there may be a plant–microbe interaction in the conversion from PL to PN within tobacco phyllosphere.

1. Introduction

Vitamin B_6 (VB_6) consists of six interconvertible pyridine compounds, which are termed “vitamers” and include pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their phosphorylated derivatives, pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP) and pyridoxal 5′-phosphate (PLP). PLP is a cofactor of enzymes catalyzing a large variety of cellular reactions mainly involved in amino acid metabolism. In recent years, additional functions of VB_6 in plants have been demonstrated as reactive oxygen species scavengers and factors able to increase resistance to biotic and abiotic stress (Ehrenshaft et al., 1999; Bilski et al., 2000). Plants are an important source of VB_6 for human and animal nutrition. The pathway for *de novo* synthesis of VB_6 has been well characterized in tobacco (Denslow et al., 2005), *Arabidopsis thaliana* (Tambasco-Studart et al., 2005; Denslow et al., 2007) and *Ginkgo biloba* (Leuendorf et al., 2008). Comparatively, knowledge about interconversion of different forms of VB_6 in plants is limited (Gerdes et al., 2012). Recently, we found an efficient conversion from PL to PN in tobacco (Huang et al., 2011a), and the enzymatic reduction of PL to PN in tobacco leaves (Huang et al., 2011b). A putative PL reductase (PLR1) was identified in *A. thaliana* based on the sequence of yeast homologue (Herrero et al., 2011). Expression of the *AtPLR1* coding region in a yeast mutant defective in PL reductase confirmed that the enzyme catalyzes the reduction of PL to PN.

PL reductase (EC 1.1.1.65), that catalyzes reduction of PL with NADPH and oxidation of PN with $NADP^+$ as the reverse reaction, was found first in budding yeast, *Saccharomyces cerevisiae*, *i.e.* bakers' (Morino and Sakamoto, 1960) and brewers' (Holzer and Schneider, 1961) yeasts. Guirard and Snell (1988) purified the enzyme to homogeneity from bakers' yeast, and found that the enzyme was a monomeric protein with molecular weight of about 33 kDa. They designated the enzyme as PL reductase because the enzymatic reaction was practically irreversible under physiological conditions. Although the conversion from PL to PN catalyzed by PL reductase in plants has been confirmed, the enzyme itself remains largely unknown. For example, PL reduction activity in a homogenate of tobacco leaves was greatly enhanced by pre-incubation in our previous study (Huang et al., 2011b). Two *Arabidopsis* T-DNA

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http://dx.doi.org/10.1016/j.plaphy.2014.10.006
0981-9428/

insertion mutant lines with insertions in the promoter sequences of *AtPLR1* were established and characterized (Herrero et al., 2011). Both plr1 mutants had lower levels of total VB_6, with significantly decreased levels of PL, PLP, PM and PMP, whereas no consistently significant change in PN and PNP levels occurred compared to wild type. These results are surprising because it has been expected that PN level would be decreased in the mutants due to the absence of PL reductase catalyzing the conversion of PL to PN. It is likely that there may be other PL reductase (s) in the VB_6 salvage pathway of *A. thaliana* that compensates for the plr1 mutation (Herrero et al., 2011).

Phyllosphere refers to leaf surface, or total above ground surface, of plants as a habitat for microorganisms. The inhabitants of the phyllosphere, termed epiphytes, consisting of a variety of bacteria, yeasts or fungi, are active members of micro-ecological system of plant phyllosphere. They have a significant role in influencing a plant's immediate environment, as well as being involved in more global processes such as carbon and nitrogen cycling (Lindow and Brandl, 2003). The ecology of epiphyte microbes is both of scientific and economical importance. In this study, a careful analysis on the enzymatic reduction of PL to PN in the homogenate of tobacco leaves indicated that the increase in the reductase activity was a consequence of phyllosphere microbial contribution. Effect of the microorganisms on the enzymatic conversion from PL to PN within tobacco phyllosphere was also analyzed using high-performance liquid chromatography (HPLC) and tracer experiment.

2. Materials and methods

2.1. Reagents, plants and enzyme samples

Pure standard B_6 vitamers were purchased from Sigma Chemicals (USA). Pyridoxal-d3 hydrochloride (^{3}H-PL) was obtained from Toronto Research Chemicals (Canada), and the chemical purity and isotopic purity of the ^{3}H-PL was 96% and 96.7% respectively. Scintillation cocktail Hisafe-3 was obtained from PerkinElmer (USA), and all other chemicals and reagents were purchased from Shanghai Sangon (China). Soil-grown tobacco (*Nicotiana tabacum cv.* Yunyan 21) was kept in a growth room at 25 ± 2 °C with a 14 h light period. Mature leaves were used for preparation of enzyme sample. By adding 2 volumes (m/v) of phosphate buffer (pH 7.4) containing stabilizing reagents (1 mM phenylmethane sulfonyl fluoride, 1 mM glutathione, 1 mM EDTA and 10% glycerol), fresh leaf tissue was completely ground using a tissue homogenizer. The homogenate was centrifuged at 12,840× *g* for 15 min at 4 °C to obtain supernatant and insoluble fraction. The homogenate was also dialyzed against in 10 mM phosphate buffer (pH 7.4) for 18 h with three changes to confirm that the enzyme catalyzes the reduction of PL with NADPH.

Fresh and young leaves were used as explants for sterile culture. Culture media for inducing callus, shoots and roots were MS+6-BA 1.0 mg/L +2,4-D 0.5 mg/L, MS+6-BA 1.0 mg/L + NAA 0.2 mg/L and MS + NAA 0.2 mg/L, respectively. The media were all complemented with sucrose 30.0 g/L and agar 8.0 g/L, and adjusted to pH 5.8. Under normal plant growth conditions (25 ± 2 °C and 14 h photoperiod at 2500 lux), culture periods were 2, 4 and 8 weeks for three different induction treatments, respectively. To encourage the plant growth, MS basal medium is usually supplemented with PN 0.5 mg/L, thiamine 0.1 mg/L and nicotinic acid 0.5 mg/L. In this study, no PN was added to the rooted medium for experiment purpose. The generated plantlets were then used for experiment. As experimental tobacco, the rooted medium was later supplemented with PL 100 mg/L. Meanwhile, some plants grown on the culture medium without the supplementation were used as control. To minimize the decomposition of VB_6 induced by light, the lower part of a plant growth flask was covered with aluminum foil. The whole operation was performed under sterile conditions. After supplementation with PL for 36 h, half of the tobacco plants were kept in an open and moist environment at 35 °C for 18 h to promote the proliferation of phyllosphere microorganisms, and the other half used as control was grown at 35 °C in sterile and closed environment.

2.2. Enzyme assay

PL reductase activity was measured according to the method described for yeast (Nakano et al., 1999) with some modifications. PL was determined by reaction with phenylhydrazine to enhance the sensitivity. Each reaction mixture (1.0 mL) contained 0.2 mM PL, 0.2 mM NADPH, 0.1 M 3-(*N*-morpholino) propanesulfonic acid/KOH buffer (pH 7.0), and the enzyme sample. The reaction was performed at 37 °C for 30 min and was stopped by the addition of 3 M $HClO_4$ (0.3 mL). The mixture was then centrifuged at 12,840× *g* for 15 min at 4 °C to remove any precipitate. To the supernatant 1 mL distilled water and 0.2 mL phenylhydrazine (0.1 M) were added, and then incubated at 60 °C for 20 min. The initial velocity of the enzyme reaction was determined by measuring the decrease in absorbance at 410 nm due to PL consumed. The enzymatic reaction was also detected by our previous method to monitor PN visually using HPLC method (Huang et al., 2011b). Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard. A unit of activity is defined as the nmol of PN formed per minute per mg of protein at 37 °C.

2.3. Extraction of microorganisms from phyllosphere

Microorganisms were extracted from tobacco phyllosphere based on the method described for bean's leaves (Zhou et al., 2006). Beef-protein medium, potato-glucose (saccharose for filamentous fungi) medium and Cause 1 medium were used to grow the microorganisms. The sampled leaves were cut in circular fragments using a sterile paper puncher. To obtain the microorganism cells from the leaf surface, disks were immersed in sterile distilled water and submitted to ultrasonic cleaner for 4 min. Cells suspensions were then used for streaking inoculation, and the colonies were selected for the amplification culture. Species identification for phyllosphere microorganisms was mostly based on morphological characteristics (cellular and colonial morphology). The culture medium with cells was used for PL reductase analysis. The microorganism cells were lysed by lysozyme and sonication, and also centrifuged at 12,840× *g* for 15 min at 4 °C to obtain supernatant and insoluble fraction. Quantity of phyllosphere microorganisms was determined by dilution method of plate counting. The CFU counts were calculated per disk surface area or per milliliter of leaf homogenate. The sampled leaves were also surface-sterilized by immersing in 75% alcohol for 1 min, then agitated in 0.01% $HgCl_2$ for 8 min, and finely rinsed three times with sterile distilled water. The germ-free leaves were completely ground with sterile distilled water to obtain endophyte. The whole operation was performed under sterile conditions. The leaf disks were also submitted in sterile distilled water, incubated at 35 °C for 2, 6 and 12 h, and then ground to investigate the relationship between PL reductase activity and microorganism quantity.

2.4. Analysis of B_6 vitamers in tobacco and its phyllosphere

B_6 vitamers were extracted from leaf tissue as follows: freshly excised leaf tissue (0.5 g) was completely homogenized with a soupcon of quartz sand, and then1.5 mL of 3 M $HClO_4$ was added and mixed by vigorous vortexing. The mixture was next centrifuged